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HIV-1 Drug Resistance and Molecular Epidemiology in Honduras

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This thesis is dedicated to my family:
To my parents, *Sergio Murillo* and *Lia Barahona*
To my brothers, *Sergio* and *Andrés*
To my beloved sister, *Nohemy*

ABSTRACT

The high genetic diversity and rapid evolution of HIV-1 poses a challenge to the worldwide prevention and treatment programs. Effective antiretroviral treatment has significantly improved the quality of life for HIV-infected patients. However, it came with a cost because resistant viruses emerge and sometimes are transmitted, which can reduce the efficacy of first-line antiretroviral therapy. Sequencing of the HIV-1 genome can provide information on both viral diversity and antiretroviral resistance profiles. This thesis work investigated HIV-1 resistance and molecular epidemiology in Honduras.

In **paper I** the prevalence of antiretroviral drug resistance was investigated in 138 HIV-positive Honduras patients with signs of treatment failure by partial sequencing of the HIV-1 *pol* gene. The prevalence of antiretroviral resistance was high and resistance mutations were detected in 112 patients (81%). Virologic failure was the strongest predictor of treatment failure and poor access to viral load testing in Honduras was identified as an important problem. **Paper II** investigated transmitted drug resistance in a representative sample of 200 treatment-naïve, newly diagnosed Honduran HIV-1 patients. The prevalence of transmitted drug resistance was 7%: 5% for NNRTI, 3% for NRTI and 0.5% for PI. Recent infection, as determined by the serological BED assay, was observed in 12% of the patients and was associated with a higher prevalence of transmitted drug resistance.

Little is known about how HIV-1 has entered and spread in Honduras and Central America. In **paper III** the molecular epidemiology of HIV-1 in Honduras was investigated using *pol* gene sequences from a representative sample of 257 Honduran patients. The Honduran HIV-1 epidemic was found to be dominated by six subtype B clades that were introduced into Honduras between 1966 and 1984. One HIV-1 clade has been particularly successful and accounts for 64% of the current HIV-1 cases in the country. The analyses suggested that HIV-1 was introduced into Honduras from the United States of America. In **paper IV** phylogenetic analyses were also used to understand the spread of HIV-1 in Central America using 625 HIV-1 *pol* gene sequences collected between 2002 and 2010 in Belize, Costa Rica, El Salvador, Honduras, Nicaragua and Panama. Published sequences from neighboring countries (n=57) and the rest of the world (n=740) were included as controls. Maximum-likelihood analyses showed that almost all (98.9%) sequences were of subtype B and that 436 (70%) sequences formed five significantly supported, monophyletic clades, which almost exclusively contained Central American sequences. One clade contained 386 (62%) sequences from all six countries; the other four clades were more country-specific, suggesting a compartmentalized epidemic. Bayesian coalescent-based methods were used to time the HIV-1 introductions and showed that the most recent common ancestor of the main subtype B introductions into Central America dated back to 1960-1970's.

In conclusion, this thesis highlights the importance of drug resistance surveillance in treated and untreated patients, and points to a need for increased access and use of HIV testing, CD4 counts, viral load and resistance testing in Honduras. Understanding the factors responsible for the HIV-1 epidemic in Honduras and Central America has important implications in terms of intervention and control strategies.

RESUMEN

La gran diversidad genética y la rápida evolución del VIH-1 desafían los programas mundiales de prevención y tratamiento. El tratamiento antirretroviral ha mejorado significativamente la calidad de vida de los pacientes infectados por VIH. Sin embargo, los virus pueden hacerse resistentes y algunas veces pueden transmitirse, lo que representa un reto en el control del VIH-1, ya que pueden reducir la eficacia de las primeras líneas de tratamiento. La secuenciación del genoma del VIH-1 proporciona información sobre la diversidad viral y los perfiles de resistencia a los fármacos antirretrovirales.

En el artículo I investigamos la prevalencia de la resistencia a los fármacos antirretrovirales en 138 pacientes hondureños VIH-1 positivos con falla terapéutica, mediante secuenciación parcial del gen *pol* del VIH-1. Los principales hallazgos demostraron que la prevalencia de resistencia a los fármacos antirretrovirales fue alta, 112 pacientes (81%) tenian mutaciones asociadas a resistencia. La falla virológica fue el principal indicador de fracaso del tratamiento y el limitado uso de las pruebas de carga viral en Honduras fue identificado como un problema importante. En el artículo II investigamos la prevalencia de resistencia transmitida en una muestra representativa de 200 pacientes hondureños VIH-1 positivos, recientemente diagnosticados y sin previa exposición a tratamiento antirretroviral. La prevalencia general de resistencia transmitida fue de 7: 5% para los INNTR, 3% para INTR y 0.5% para los IP. Se determinó, mediante la prueba de incidencia BED, que 12% de los pacientes tenían infecciones recientes, y fueron asociadas con una mayor prevalencia de resistencia transmitida.

Poco se sabe como el VIH-1 entró y se extendió en Honduras y Centro América. En el artículo II estudiamos la epidemiología molecular del VIH-1 en Honduras usando secuencias del gen *pol* en una muestra representativa de 257 pacientes hondureños. Encontramos que la epidemia de VIH-1 en Honduras es dominada por al menos seis cepas del subtipo B que se introdujeron al país entre 1966 y 1984. Una cepa de VIH-1 fue particularmente exitosa y representa el 64% de los actuales casos de VIH-1 en el país. Los análisis sugieren que el VIH-1 se introdujo en Honduras vía Estados Unidos. En el artículo IV, usamos los análisis filogenéticos para comprender la propagación del VIH-1 en Centro América. Utilizamos 625 secuencias del gel *pol* colectadas entre el 2002 y 2010 en Belice, Costa Rica, El Salvador, Honduras, Nicaragua y Panamá. Los análisis mostraron que casi todas las secuencias (98.9%) eran subtipo B y que 436 (70%) secuencias estaban agrupadas en cinco grupos monofiléticos estadísticamente significativos, formados exclusivamente por secuencias de Centro América. Un grupo contenía 386 (62%) secuencias de los seis países centroamericanos, los otro cuatro grupos eran más específicos de cada país, lo que sugiere una epidemia compartimentalizada. Se estimó que la introducción del HIV-1 subtipo B en Centro América se remonta entre los años 1960 y 1970.

En conclusión, este trabajo muestra la importancia de la vigilancia de la resistencia del VIH-1 a los fármacos antirretrovirales en pacientes tratados y no tratados, de aumentar el uso de las pruebas de VIH, el recuento de CD4, la carga viral y las pruebas de resistencia en Honduras. Entender los factores responsables de la epidemia de VIH-1 en Honduras y Centro América tiene importantes implicaciones en términos de intervención y estrategias de control.

LIST OF PUBLICATIONS

I. **Murillo W**, de Rivera IL, Parham L, Jovel E, Palou E, Karlsson AC, Albert J. Prevalence of drug resistance and importance of viral load measurements in Honduran HIV-infected patients failing antiretroviral treatment. 2010. HIV Med. 11:95-103.

II. **Murillo W**, Paz-Bailey G, Morales S, Monterroso E, Paredes M, Dobbs T, Parekh BS, Albert J, Rivera IL. Transmitted drug resistance and type of infection in newly diagnosed HIV-1 individuals in Honduras. 2010. J Clin Virol. 49:239-44.

III. **Murillo W**, Skar H, Lorenzana de Rivera I, Paz-Bailey G, Morales-Miranda S, Albert J, Mild M. Molecular Epidemiology of HIV-1 in Honduras. Manuscript.

IV. **Murillo W**, Veras N, Prosperi M, Lorenzana de Rivera I, Paz-Bailey G, Morales-Miranda S, Marín JP, Pascale JM, Mild M, Albert J, Salemi M. One early HIV-1 subtype B introduction into Central America around 1966 accounts for most current cases. Manuscript.

The papers will be referred to in the text by their Roman numerals.

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
PBMCs	Peripheral blood mononuclear cells
cART	Combination antiretroviral therapy
CDC	Centers for Infections Disease Control and Prevention
CRFs	Circulating recombinant forms
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
FSW	Female sex workers
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PAHO	Pan American Health Organization
PI	Protease inhibitor
RNA	Ribonucleic acid
SIV	Simian immunodeficiency virus
STDs	Sexual transmitted diseases
UNAH	Universidad Nacional Autónoma de Honduras
URFs	Unique recombinant forms
UNAIDS	Joint United Nation Program on HIV/AIDS
WHO	World Health Organization

CONTENTS

1 HIV

1.1 THE HIV PANDEMIC

1.1.1 The global epidemiology of HIV

HIV has spread worldwide. Since the beginning of the epidemic, more than 60 million people have become infected with HIV and more than 25 million have died of AIDS-related causes [1]. At the end of 2010, WHO estimated that there were 34 million people living with HIV; 3.4 million of them were children under the age of 15. The same year, 2.7 million people became newly infected with HIV, which means that more than 7,000 and 300 people contracted HIV every day and hour, respectively. Furthermore, 1.8 million people were estimated to have died from AIDS in 2010 [2].

Although HIV and AIDS are found in all parts of the world, some areas are more afflicted than others. The majority of the people living with HIV/AIDS live in low- and middle-income countries. Sub-Saharan Africa is the hardest-hit region and is home to 67% of all people living with HIV worldwide, where in a few countries more than one in five adults are infected with HIV. Outside sub-Saharan Africa, the Caribbean has the highest HIV prevalence. Parts of Asia and Latin America are also experiencing severe epidemics at the national or local level. The epidemic is spreading most rapidly in Eastern Europe and Central Asia, where the number of people living with HIV increased by 54.2% between 2001 and 2009 [1].

In high-income nations, HIV infections have historically been concentrated principally among injecting drug users and men who have sex with men (MSM). These groups are still at high risk, but heterosexual intercourse accounts for a growing proportion of cases. In the United States, a quarter of people diagnosed with AIDS in 2008 were female, and three quarters of these women were infected as a result of heterosexual sex [3]. In several countries in Western Europe, including the United Kingdom, heterosexual contact is the most frequent cause of newly diagnosed infections, but many of these persons are immigrants from high-endemic countries in Africa and Asia [4].

1.1.2 HIV and AIDS in Central America and Honduras

1.1.2.1 General information of Central America

Central America is the isthmus which connects North America with South America, and consists of seven countries: Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama (Figure 1). Central America is considered to be part of the

developing world. In general, the standard of living of the general population in the Central American countries tend to be below the poverty line which translates into a shortened life span, higher illiteracy rates and lower quality of the health care. There is significant economic diversity between the countries in Central America. Nicaragua is considered the poorest and the least developed of the Central American countries, followed by Honduras. In contrast, Panama and Costa Rica are more developed. With respect to GDP per capita, Panama ranks highest with a purchasing power parity of US$13,000; however Costa Rica has the highest development level due to a relatively high GDP per capita and the best demographics indicators of the Central American countries [5].

The Central American countries are primarily agricultural. Thus, relatively high proportions of the population have low income and make their living on traditional agriculture, characterized by low-level technology and intensive manual work. Unemployment levels are higher than 20% and are an important reason for poverty, especially in the rural population. The major cities of Central America have experienced rapid urban growth in recent decades due to the poor economical conditions in the rural regions. Temporary employment is a common feature in most cities in Central America, but few cities can support the increasing demand for jobs, forcing the inhabitants of these countries to migrate legally or illegally to other countries, especially to the United States in search of better economic possibilities.

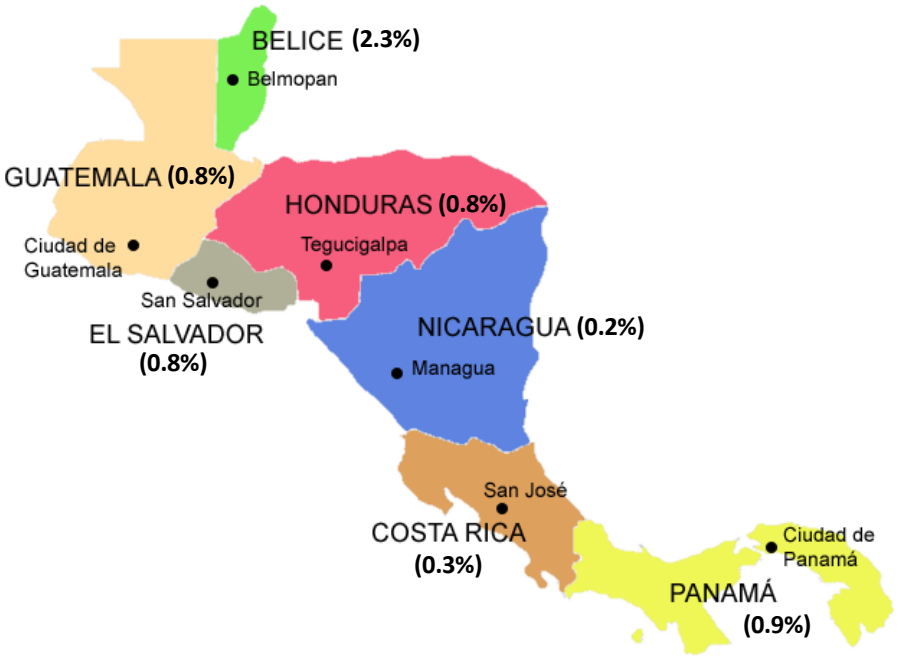

Figure 1. Political division of Central America. The map shows the seven Central American countries, the dots highlight the capitals cities. HIV prevalences are shown in parentheses.

1.1.2.2 HIV/AIDS epidemic in Central America

The HIV/AIDS epidemic in the Central America region is characterized by the poorest countries having the highest adult HIV prevalences [6]. The HIV prevalence among adults in the Central American countries ranges from an estimated 0.2 to 0.9 percent, except for Belize which has an estimated prevalence of 2.3%. Nicaragua and Costa Rica have the lowest prevalence, 0.2% and 0.3%, respectively [7, 8]. WHO estimates that approximately 200,000 people in Central America live with HIV, with Guatemala, Honduras and El Salvador contributing the largest number of cases [9-11]. The first cases of HIV in Central America were reported among MSM in the middle 1980's, but the virus has spread primarily in the general population through heterosexual contacts [12]. Thus, heterosexual transmission accounts for 70% of the cases even though HIV-1 prevalence is still higher among MSM, female sex workers (FSW), prisoners, and some ethnic groups [1]. The prevalence among MSM varies from country to country and has been reported to be as high as 15% in Guatemala, 12.7% in Costa Rica, and 10.8% in El Salvador [13-15]. The HIV prevalence among FSW has been estimated to be 4.1% in Honduras, 4.3% in Guatemala and 5.7% in El Salvador [11, 13-16]. Information on the impact of the epidemic in ethnic groups is limited, but the prevalence of HIV infection appears to be high (around 4.5%) in the Kuna population in Panama and the Garifuna population in Belize, Guatemala and Honduras [17]. There are several reasons why the Kuna ethnic group, descendants of the Carib Indians, has high HIV prevalence is that they generally lack information about STIs and HIV/AIDS, and most of them do not use condoms or even know what condoms are. Sexual activity for Kuna women typically begins between 11 and 15 years of age, with males starting slightly later. Males are initiated in sexual intercourse by the "*omegit"* (a male transsexual group that are perceived as being women) [18]. The spread of the epidemic through Garifuna communities, an ethnic group descended from African slaves and Carib Indians, is likely facilitated by cultural patterns of multiple sexual partnerships among men and women [17, 19].

According to limited available HIV surveillance data, HIV/AIDS epidemic in Central America is concentrated in large urban areas, with highest prevalence rates in some areas along the Caribbean coast [7]. The main risk factors for HIV infection in the region are: early sexual debut; social pressure for male to have multiple sexual partners; widespread poverty; women's and girl's inability to negotiate when and under what circumstances to have sex or use of condoms [7]. Another factor that contributes to the HIV epidemic in Central America is a large degree of mobility and migration. Migration can be due to temporary jobs within the Central American countries or looking for permanent jobs in the United States [20, 21]. While in transit, migrants seem to be engaged in high-risk sexual behavior that contributes to the widespread of the disease.

1.1.2.3 HIV/AIDS epidemic in Honduras

In Honduras the first case of AIDS was identified in 1984 in a resident of El Progreso city, a city in the North region of Honduras, who reported having made several trips to San Francisco in the United States in the previous years to his illness. His HIV diagnosis was made in 1985. After this report, four additional men were identified as HIV infected; three of them were classified as homosexual transmission and one as a heterosexual transmission. All four had a history of traveling abroad [22]. In the report "Analysis of the evolution of the epidemic in Honduras, in 1998" the authors present an analysis of the first 100 cases of AIDS in the country. The male/female ratio was 2:1. Most affected persons were between 26 and 30 years of age. Among the heterosexuals, 11 were FSW and 2 were men who reported using intravenous drugs. Sixty-one percent of heterosexuals reported having more than one sexual partner in the last 5 years. A total of 18 were classified as homosexual transmission and 15 cases as bisexual transmission. Only one case reported blood transfusion. Finally, there were two cases of pediatric AIDS, both cases from infected mothers. Among the 90 people with known residence, 67 lived at the North Coast, and 34 of them lived in San Pedro Sula.

Table 1. HIV and AIDS in Honduras. Updated June 2011 [10].

Description	n	
The HIV and AIDS epidemic		
Estimated prevalence	39,000	(0.8%)
Cumulative HIV positive cases	29,597	
Cumulative AIDS cases	21,330	
Asymptomatic cases	8,267	
Male	15,577	
Female	14,020	
Children under 15 years	1,929	
Antiretroviral therapy		
In need of cART*	21,000	
Receiving cART	8,018	
Adults	7,291	
Children	727	
In 1st or 2nd line therapy	8,004	(99.8%)
In salvage therapy	14	(0.2%)

*In need of combination antiretroviral therapy based on WHO 2010 guidelines

Since the first HIV case in Honduras was reported, more than 29,000 cases have been diagnosed (table 1) [10]. Today Honduras has an estimated adult HIV prevalence of 0.8% [0.5% - 1%], which corresponds to 39,000 infected persons [1]. It is estimated more than 2,500 HIV-infected Honduran have died from AIDS. HIV in Honduras is mainly transmitted by unprotected sex, and the pattern of transmission is

predominantly heterosexual. Prevalence rates among MSM, FSW and Garifunas are significantly higher than in general population, 9.9%, 5% and 4.5%, respectively [13, 23]. Geographically, the prevalence is higher along the north coast compared to the remaining parts of the country [10, 24].

1.1.3 The discovery of HIV

The very first step in the discovery of HIV was the observation of AIDS-related illnesses and deaths. The new disease was formally recognized on June 5, 1981 when the Centers for Disease Control and Prevention (CDC) reported that five Los Angeles men had developed an unexplained immunodeficiency [25]. Additional cases were soon reported so that in early 1980s, there was an increase in the incidence of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma in previously healthy young homosexual men in New York and California [26-31]. After these reports, AIDS was found among intravenous drug users [32], Haitians living in the United States [33], hemophiliacs [34], transfusion recipients [35], infants [36], and female sexual partners of males with AIDS [37]. Additional reports from Europe suggested that the AIDS epidemics had two faces. In the United Kingdom, West Germany and Denmark, the majority of people with AIDS were homosexual, and many people had a history of sex with North American men. However, in France and Belgium AIDS was occurring mainly in people from Central Africa or those with links to that area [38-40].

Based in all these finding, the acronym AIDS, which stands for acquired immunodeficiency syndrome, was coined in 1982 [41]. By this time, it was evident that the disease was transmitted by blood contact, sex, and from mother-to-child. In May 1983, doctors at the Institute Pasteur in France reported that they had isolated a new virus retrovirus, which they suggested might be the cause of AIDS [42]. A year later the association with AIDS was confirmed by researchers at the National Cancer Institute in the United States [43]. The new retrovirus was called human immunodeficiency virus (HIV) [44]. In 1986, a related, but distinct, retrovirus was isolated from West African individuals and named HIV-2 [45, 46].

1.1.4 The origin of HIV

After HIV was discovered, HIV-related non-human primate retroviruses were identified and named simian immunodeficiency viruses (SIV) [47, 48]. More than 40 different primate species, all originating from Africa, have been shown to be infected with SIV, including African green monkeys, sooty mangabeys, mandrills, chimpanzees, and gorillas (Figure 2). HIV is the result of multiple cross-species transmission of a few of these SIVs. It is now well established that West Central African chimpanzees (*Pan troglodytes troglodytes*) are infected with SIVcpz*Ptt*, which has been introduced to humans and is now recognized as HIV type 1 (HIV-1) [49-52]. HIV-1 is not just one

virus, but comprises four distinct lineages, termed groups M, N, O and P. HIV-1 groups M and N are very closely related to SIVcpz*Ptt* strains from southern Cameroon [52]. Recently, SIVgor was discovered among western lowland gorillas (*Gorilla gorilla gorilla*) [53-55]. Phylogenetic data suggest that HIV-1 group O and P are genetically related to SIVgor [56]. However, chimpanzees are more likely the original reservoir also of these SIV strains (Figure 3).

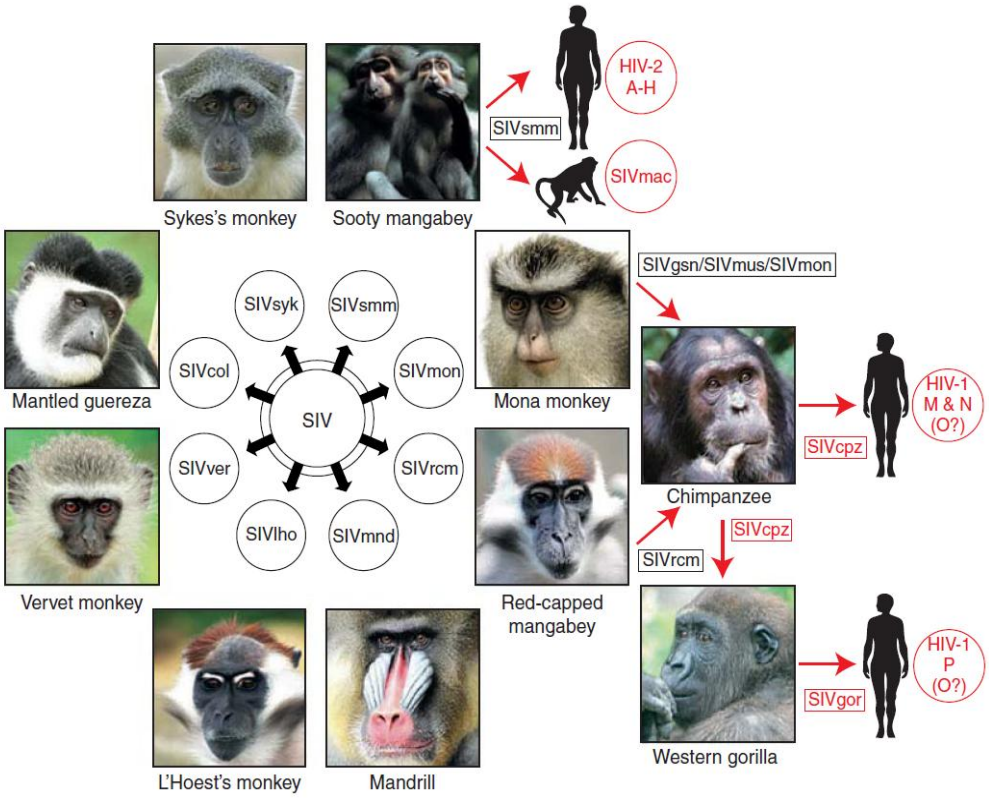

Figure 2. Origins of human AIDS viruses. Simian immunodeficiency viruses (SIVs) with a suffix to denote their primate species of origin (e.g., SIVsmm from sooty mangabeys). Several of these SIVs have crossed the species barrier to great apes and humans, generating new pathogens. Known examples of cross-species transmissions, as well as the resulting viruses, are highlighted in red. *Reprinted with permission* [57].

In 1989, a closely related SIV (SIVsmm) was found in sooty mangabey monkeys (*Cercocebus atys atys*) [58, 59], and subsequently confirmed by demonstrating that humans in West Africa harbored HIV-2 strains that resembled locally circulating SIVsmm infections [59, 60]. Since HIV-2 was isolated for first time in 1986, at least eight different lineages have been identified, each of which appears to represent an independent cross-species transmission (Figure 4).

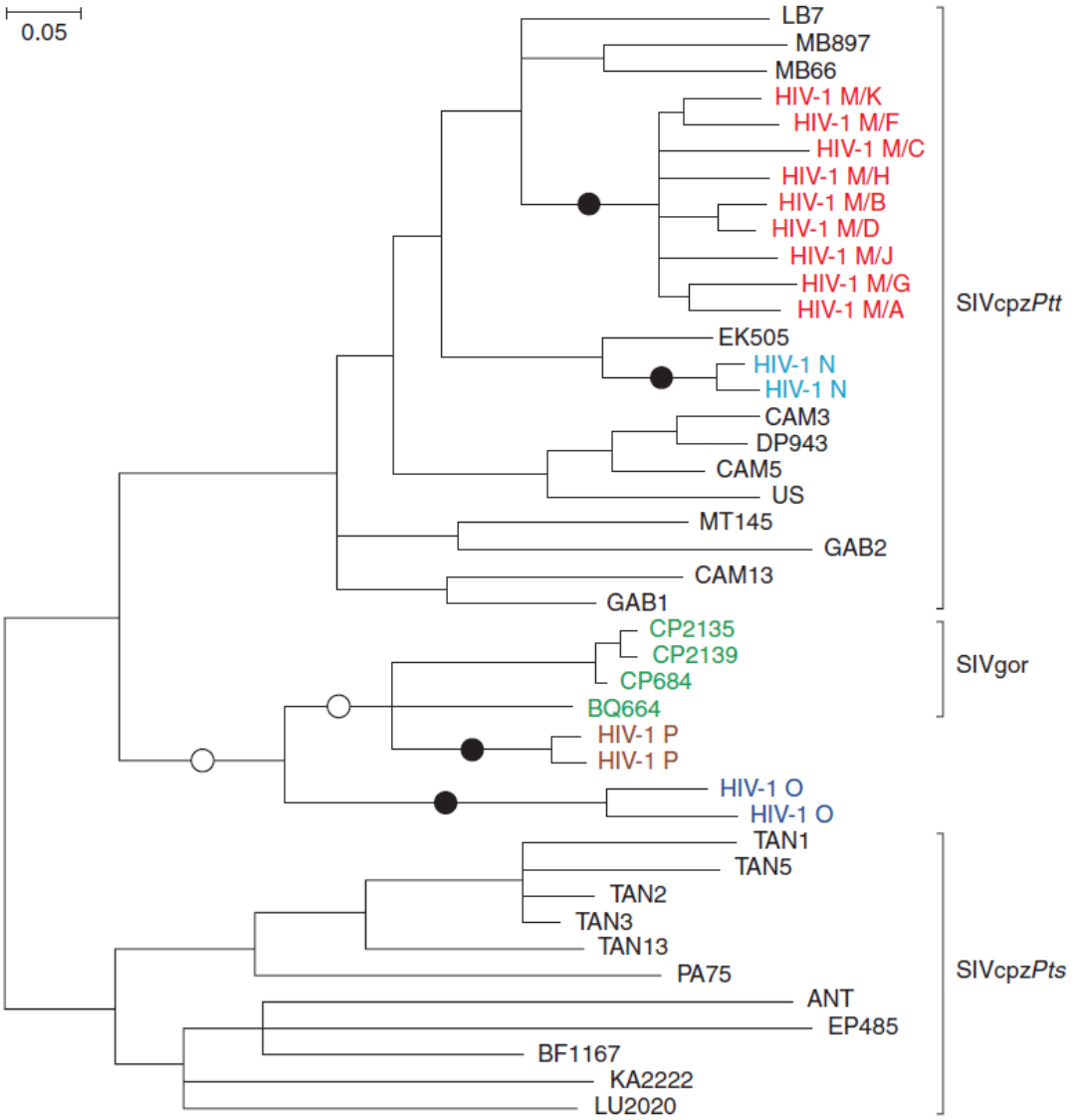

Figure 3. A maximum likelihood phylogenetic analysis of HIV-1, SIVcpz and SIVgor. The tree was reconstructed using *pol* gene sequences (HIV-1/HXB2 coordinates 3887–4778). SIVcpz and SIVgor sequences are shown in black and green, respectively. The four groups of HIV-1 are shown in different colors. Black circles indicate the four branches where cross-species transmissions to humans have occurred. White circles indicate two possible alternative branches on which chimpanzee-to-gorilla transmission occurred. Brackets at the right denote SIVcpz from *P. t. troglodytes* (SIVcpzPtt) and *P. t. schweinfurthii* (SIVcpzPts), respectively. The scale bar represents 0.05 nucleotide substitutions per site. *Reprinted with permission* [57].

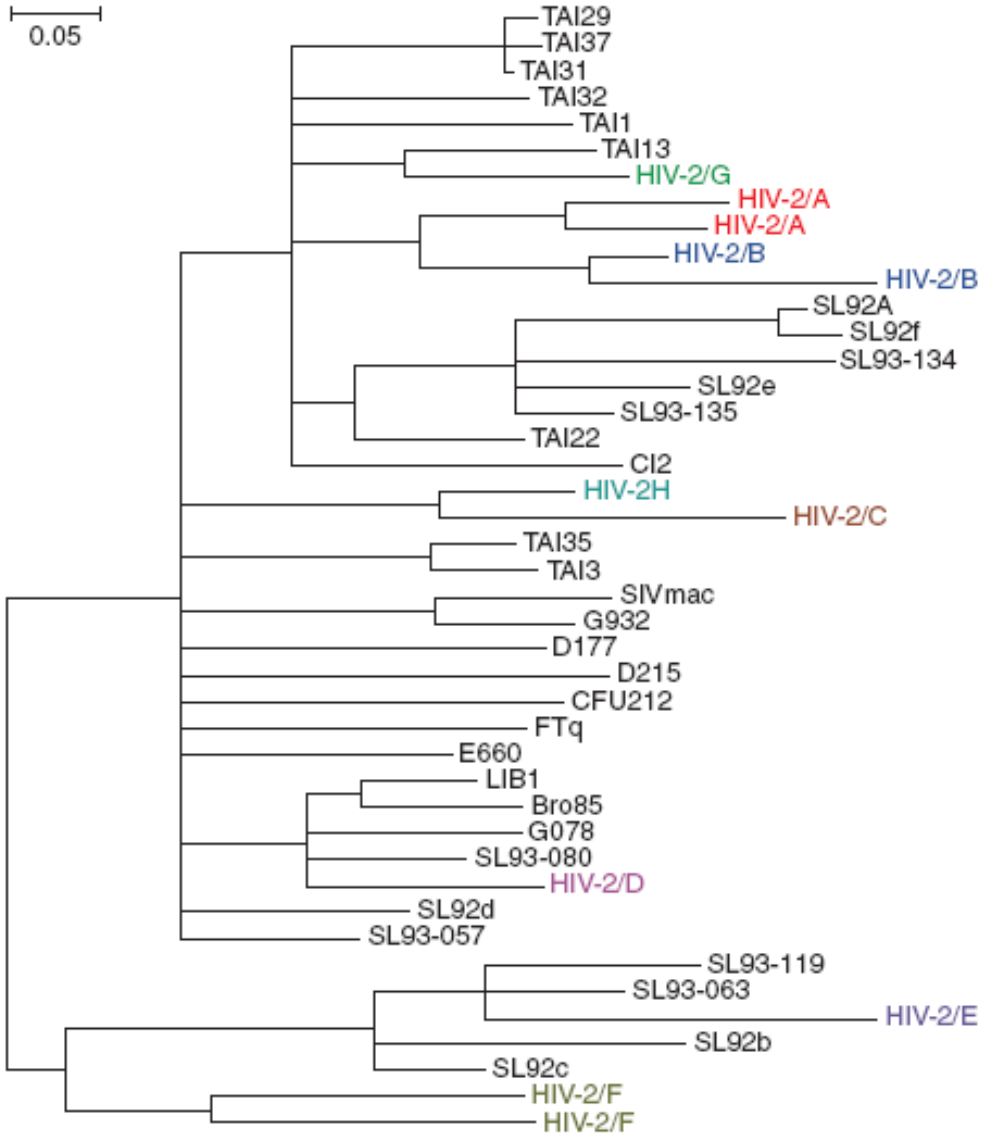

Figure 4. A maximum likelihood phylogenetic analysis of HIV-2 and SIVsmm. The tree was reconstructed using *gag* gene sequences (SIVmac239 coordinates 1191–1921). SIVsmm and SIVmac are shown in black; the eight groups of HIV-2, each of which represents an independent cross-species transmission, are shown in different colors. The scale bar represents 0.05 nucleotide substitutions per site. *Reprinted with permission* [57].

1.2 HIV VIROLOGY

1.2.1 Structure

HIV belongs to the *Retroviridae* family and the *Lentivirinae* genus. The virion is spherical, enveloped, with a diameter of approximately 120 nm, and contains two copies of positive-sense, single-stranded RNA molecules (Figure 5).

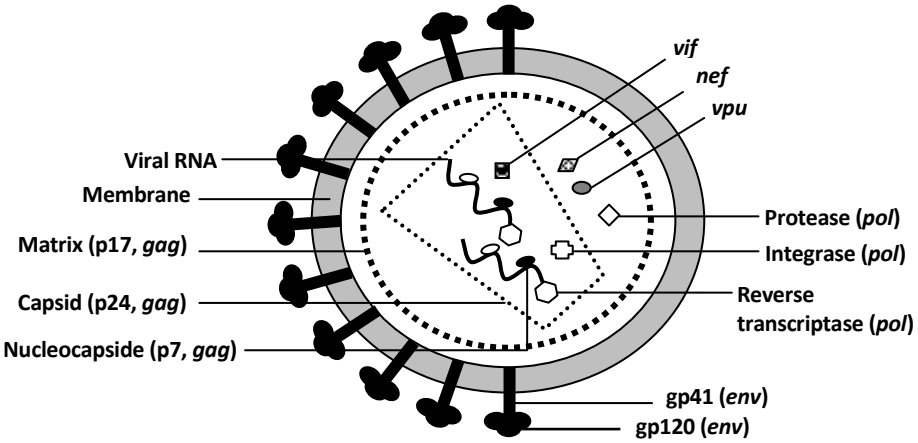

Figure 5. Schematic structure of the HIV-1 particle.

The HIV genome is approximately 10 kilobases in length. Like other retroviruses, the HIV genome has three major structural genes: group-specific antigen (*gag*), polymerase (*pol*), and envelope (*env*). Additionally, HIV has regulatory genes: transactivator of viral transcription (*tat*), regulator of RNA transport (*rev*); and accessory genes: viral infectivity factor (*vif*), viral protein R (*vpr*), negative factor (*nef*), and the viral protein U (*vpu*) for HIV-1 or viral protein X (*vpx*) for HIV-2. The nine genes are flanked by two repetitive regions called non-coding long terminal repeats (LTRs). Both LTRs are needed for the incorporation of the viral genome into the host cell genome. Figure 6 summarizes the important functions of HIV-1 genes.

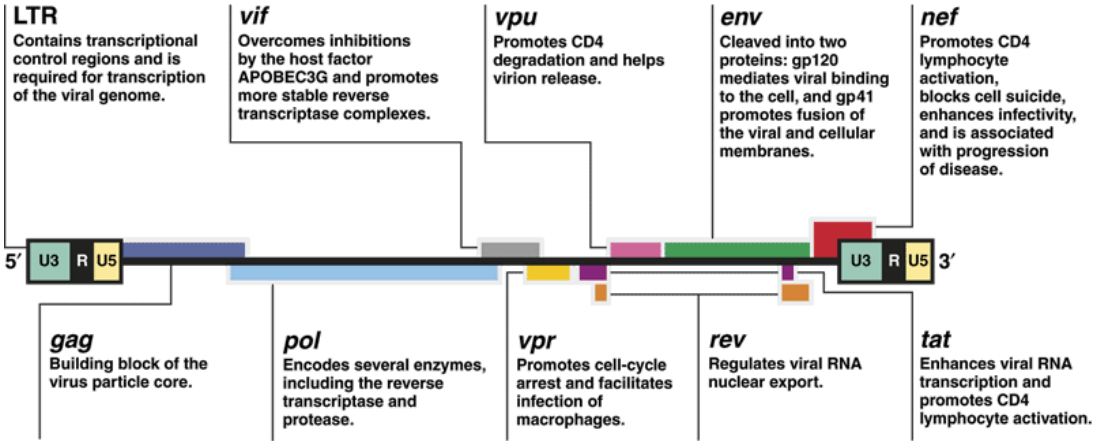

Figure 6. Genomic organization of HIV-1. Summary of the major functions of the 9 structural, regulatory and accessory genes. Courtesy of Gladstone Institute [61].

1.2.2 Replication

HIV infects cells of the immune system that have CD4 receptors on their surfaces [62, 63]. These cells include T-lymphocytes, monocytes, macrophages, dendritic cells and brain microglia. The initial step of the HIV infection involves binding of the viral gp120 glycoprotein to the CD4 receptor. This binding induces a conformational change in gp120, and enables gp120 to interact with the co-receptor CCR5 or CXCR4 [64]. Binding to the co-receptors brings the virion in close proximity to the cellular membrane, allowing a part of the gp41 to penetrate the cell membrane. This binding let the virus to fuse with the cell (Figure 7).

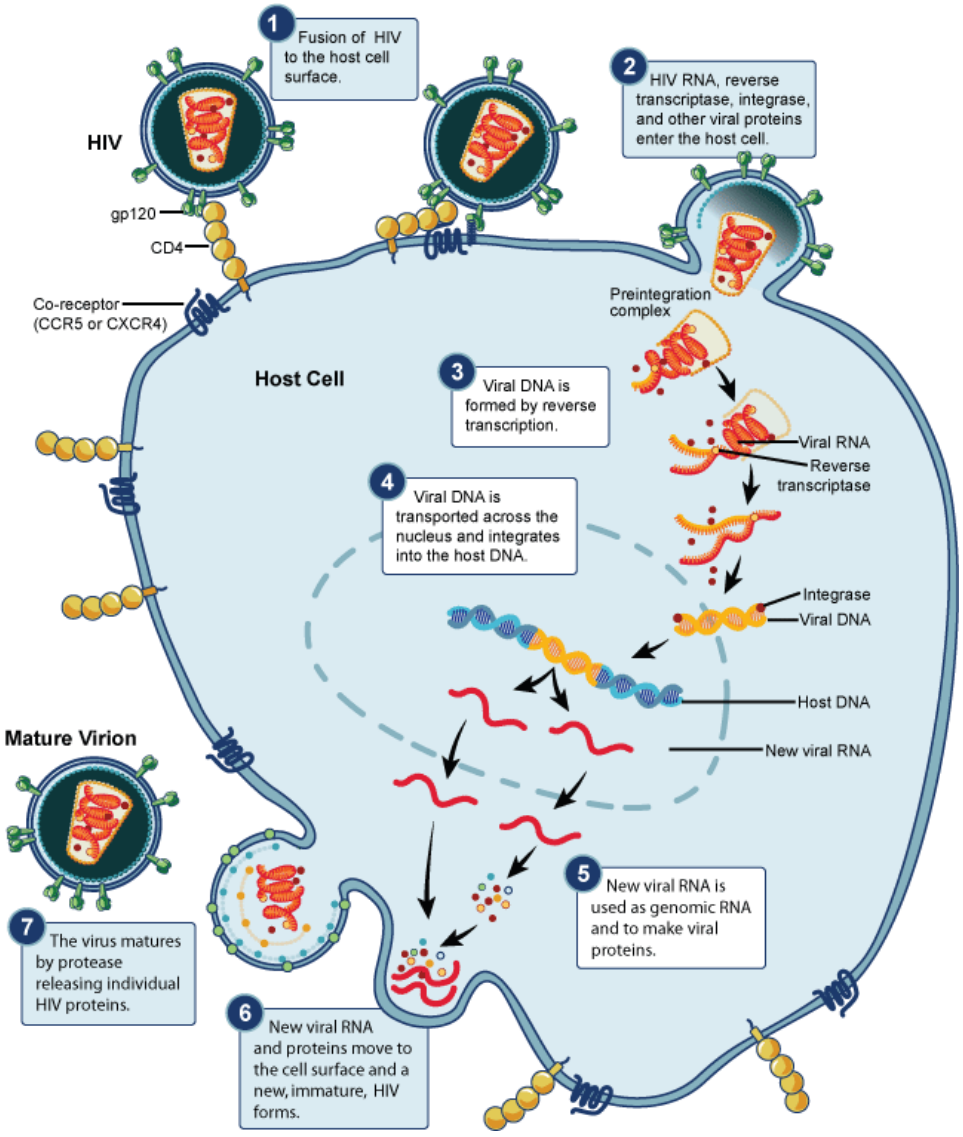

Figure 7. HIV replication cycle.

Courtesy: National Institute of Allergy and Infectious Diseases (NIAID)

After the viral and cell membranes have fused, the viral nucleocapsid, which contains the viral RNA genome, is released into the cytoplasm, where the reverse transcriptase is activated and begins synthesizing viral cDNA. The pre-integration complex is subsequently transported to the cell nucleus, where the HIV integrase catalyses the integration of the viral cDNA into the host-cell genome. The integrated viral DNA is referred to as provirus. Once integrated into the host-cell genome, the transcription activity of the HIV provirus is regulated by constitutive host-cell transcription factors [65]. The replication of the viral genome begins with the transcription of the proviral DNA into a primary RNA transcript, which serves both as genetic material for new virions as well as mRNA for HIV protein synthesis. Several different classes of HIV mRNA are produced through alternative splicing. These viral mRNAs contains the code to produce the structural and auxiliary proteins for viral replication and assembly. The viral envelope proteins are glycosylated and are produced in the rough endoplasmic reticulum, and then move to through the Golgi apparatus before arriving at the cell surface. Viral RNA molecules, along with the other viral components, assemble at the membrane and bud off from the host cell.

1.3 HIV GENETIC VARIATION

1.3.1 Sources of variation

A remarkable characteristic of HIV is the enormous capacity of genetic variation and the rapid evolution. Studies on HIV-1 have determined that a combination of multiple mechanisms contributes to the high genetic variability of the virus, including:

1. The viral replication rate in vivo is very rapid all along the infection, with an estimated production of 10^{10} new virions each day and a mean generation time of 2–3 days [66-68].
2. The mutation rate is very high and is derived from an error-prone viral reverse transcriptase, which introduces mutations at an average of approximately 0.1-0.3 per genome per replication cycle [69-71], which remains uncorrected because of the lack of proof-reading activity of the viral enzyme.
3. The recombination rate is also very high. Recombination occurs because reverse transcriptase copies the two RNA molecules packaged into the virion alternatively, generating a mosaic DNA genome that is the source of progeny virions. The crossover frequency ranges from 7 to 30 per replication round [72, 73].

There are also other factors that contribute to HIV genetic variation. The majority of the mutations are caused by the viral reverse transcriptase. However, it has been determined that host RNA polymerase II, active during the late stage of viral replication from proviral DNA to progeny viral RNA, contributes to retroviral mutations [74, 75]. Human apolipoprotein B mRNA-editing enzyme catalytic

polypeptide-like (APOBEC) is an innate virus restriction factor that inhibits HIV-1 replication and induces excessive deamination of cytidine residues in nascent reverse transcripts. Specifically APOBEC3G introduces G-to-A mutations in the viral RNA, contributing to the diversity and evolution of HIV-1 [76].

Due to the processes above mutations and recombinants are constantly generated. Many mutations are deleterious and will be removed by purifying selection, but positive Darwinian selection is also in operation. Thus, the immune system plays an important role on HIV diversification and evolution [77, 78]. During primary infection, prior to the induction of an immune response the viral population is usually highly homogeneous [79]. Subsequent diversification is strongly influenced by immune selection, with genetic diversity increasing linearly during the period of immune competence. Eventually, lower diversification rates develop at late stages, coinciding with the decline of efficient immune responses [80].

1.3.2 Genetic classification

Based on genetic similarities, HIV is classified into types, groups, subtypes, circulating and unique recombinant forms (CRFs and URFs, respectively). HIV is first classified into two types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 was isolated for first time from French and US patients in 1983-1984 [42, 81, 82] and HIV-2 was isolated in 1986 from patients in West Africa [45]. Both types of virus are morphologically and biologically similar, but only present 42% homology in their genomes [47]. HIV-1 is classified into four groups, M (main), O (outlier) N (non-M-non-O) and P (Figure 3). These four groups resulted from four separated introductions of SIV into humans (*Section 1.1.4*). Group M was the first to be discovered and to be studied extensively. In the early 1990's a new strain of HIV-1 was isolated from two Cameroonian patients living in Belgium [83]. The virus was divergent from the known HIV strains, but clustered more closely with HIV-1 (65-79% sequences homology) than with HIV-2 (56% homology). After more cases were identified, the virus was classified as HIV-1 group O [83-85]. Group O viruses represent less than 1% of the global HIV-1 infections and are largely restricted to Central Africa, mainly Cameroon and Gabon and neighboring countries [86-88]. Group N was discovered in 1998 [89], and is less prevalent than group O. So far few cases of HIV-1 group N has been documented, all, except one [90], in individuals from Cameroon [91, 92]. Finally, group P was discovered recently in two Cameroonian persons [56, 93].

The vast majority of HIV infections globally belong to HIV-1 group M. Within group M nine genetically distinct subtypes have been described: A, B, C, D, F, G, H, J and K (Figure 3) [94-102]. The subtypes differ by up to 30% in the *env* gene, 20% in *gag* and 15% in *pol* [103, 104]. Even within subtypes there is significant diversity. For example, subtype A and F are further divided into sub-subtypes, A1 through A3 [105-107] and

F1 and F2 [108]. Recombination plays an important role in the HIV-1 pandemic. More than 50 CRFs have been officially recognized so far (http://www.hiv.lanl.gov). The CRFs arise from recombination of two or more subtypes; they are numbered sequentially and named according to their subtype composition. For example, one recombinant of subtype A and G is labeled CRF02_AG [109]. When recombination involves three or more strains, the designation "cpx" for "complex" replaces the subtype notation. For example, one recombinant of subtypes A, G, J and K is named CRF06_cpx [104]. CRF01_AE and CRF04_cpx were initially named subtype E and I, but were subsequently found to be recombinants [110-114]. In addition to the CRFs, there are also numerous of URFs, particularly in areas with high HIV diversity [115].

HIV-2 was found, and is still most common, among individuals from West Africa. At least eight distinct lineages of HIV-2 have been identified, each of which appears to represent an independent cross-species transmission that occurred in West Africa. These lineages are termed groups A-H, although only groups A and B have spread within humans to an appreciable degree (Figure 4) [116].

1.3.3 Significance of genetic variation

The pathogenesis of the two major genetic types of HIV has been shown to differ significantly, with HIV-2 being both less transmissible and less pathogenic than HIV-1 [117-119]. Several studies have reported differences in co-receptor usages between the different HIV-1 subtypes [120-123] that affect in tissue tropism, transmission and disease progression [124-129]. Some published studies have suggested that HIV-1 subtypes or CRFs may differ in efficiency of transmission. One mother-to child transmission study showed that subtype C was transmitted more frequently than subtype B [127]. In another study pregnant women from Kenya infected with subtype C were more likely than those infected with subtype A or D to shed HIV-1-infected vaginal cells, implying that sexual transmission may be more likely with this subtype [130]. A longitudinal cohort study of injection-drug users in Thailand found an increased probability of transmission of CRF01_AE as compared with subtype B [126].

There have also been several studies that report differences in disease progression. An early study in 1999 by Kanki *et al.* reported that patients infected with subtype C, D and G were more likely to develop AIDS compared to patients with subtype A [128].The same year Neilson *et al.* suggested that subtype C conferred more rapid disease progression that subtype A and D [131] and in 2001, Kaleebu *et al.* published that subtype D gave a more rapid disease progression that subtype A [132]. As noted, these three studies do not agree completely with each other. Subsequently studies have reported discordant results. In one early Swedish study of patients infected with subtypes A, B, C, or D, no significantly differences in diseases progression were detected [133].

With regards to response to therapy, some HIV-1 subtype-specific patters of drug mutations have been reported [134-136]. Frater *et al.* found no significant differences in the response to therapy among patients infected with subtype A, C or D [137]. Similar findings were shown in a study that included children infected with subtypes A, B, C, D, F, G, H, E/D and A/G [138]. Another study found that isolates of subtype D had a slightly lower susceptibility to antiviral drugs, but suggested that this might be explained by the more rapid growth rate that was found in these isolates [139]. Overall, it appears that HIV-1 subtypes do not show major differences in the response to antiretroviral therapy. However, there are some data that indicates the effect of subtypes in diagnostic tests [140, 141] and in the development of vaccines [142, 143].

In summary there is still uncertainty about the biological and clinical significance of HIV diversity. In routine patient management it is rarely necessary to consider the subtype of the patients' virus. However, subtypes may influence the accuracy of laboratory diagnostics and monitoring of HIV-1 infection, especially when molecular tests are used [144-148]. Furthermore, it is important to study and classify the different variants of HIV to track the course of the global spread of the virus.

1.3.4 Phylogenetics

Phylogenetics is the scientific discipline concerned with describing and reconstructing the patterns of genetic relationships among species. Phylogenetic analyses may also be used to study the origin and to follow the evolution of rapidly changing species such as RNA viruses. In phylogenetic studies, the most convenient way of visually presenting evolutionary relationships among a group of organisms or individuals of the same species is through illustrations called phylogenetic trees.

Modern phylogenetic and phylodynamics methods that incorporate the coalescent theory of population genetics have been developed. These high-resolution phylogenetic approaches have been used to evaluate the origin, evolution, demographics and migrations patters of HIV-1 in several countries [149-155]. Thus, phylogenetic studies have indicated that HIV-1 and HIV-2 entered the human population through multiple zoonotic infections from SIV-infected non-human primates (*See section 1.1.4. about the origin of HIV*). The extraordinary global genetic diversity of HIV-1 has been used to classify viral variants based on their phylogenetic relationship [104] (*See section 1.3.2. about the genetic classification of HIV*).

Phylogenetics approaches are increasingly used to examine transmission networks of HIV [156]. Hué *et al.* suggested that the HIV-1 subtype B epidemic in the UK was comprised of at least six established chains of transmission [157]. In the phylodynamics reconstruction of the HIV transmission network among men who sex

with men in London, Lewis *et al.* found that the HIV-1 epidemic in this population has been episodic [158]. Skar *et al.* found multiple HIV-1 introductions into the Swedish intravenous drug user population and also characterized the dynamics of spread of different variants [159].

Phylogenetic reconstruction has also been used to date the origin of the HIV epidemic. It has been estimated that HIV-1 group M originated around 1910 in Central West Africa [160], and the origin of HIV-2 infection in humans to around 1940 in West Africa [161]. Gilbert *et al.* shown that subtype B, which account for most of the HIV-1 infections in the Americas and Europe, moved from Africa to Haiti around 1966 (1962-1970) and then onward to the US and other parts of the world [162]. The date of the origin US HIV-1 epidemic has been estimated around 1968-1969 [162, 163]. Extending these data, Junqueira *et al.* (2011) recently investigated the role of South American countries in the evolutionary history of subtype B in the Americas, considering the close economic, historical and social relationship of the Caribbean countries with South American countries. Junqueira *et al.* provided evidence of a direct introduction of non-pandemic subtype B, from Caribbean countries, not only Haiti, into northern South America early in the epidemic. The analysis also showed a phylogenetic link of pandemic subtype B variants from North and South America. They also found a relationship of South American and Mexican sequences. Based on this and the large migration of Mexicans to the United States, they suggested that Mexico could have represented the entrance door for the epidemic into the United States. These two studies have provided important information about the evolutionary history of HIV-1 subtype B in the Americas, but still very little is known about the epidemic in Central America. This lack of information about HIV-1 spread in Central America was the basis for papers III and IV of this thesis.

1.4 HIV INFECTION

1.4.1 Transmission

The three main routes of human-to-human HIV transmissions are: sexual intercourse, blood products, and mother-to-child transmission. It is estimated that HIV sexual transmission (heterosexual or homosexual) stands for more than 80% of all global HIV infections. Heterosexual transmission is the major route of transmission and accounts for 60-70% of the cases [1]. As HIV is present in blood, transmission can occur via blood transfusion or blood products, intravenous drug use, or accidents in health care or laboratory work. Mother-to-child transmission is responsible for more than 90% of pediatric infections worldwide. Children can be infected during pregnancy, *in utero*, during delivery or postnatally through breastfeeding. In the absence of treatment, the transmission rate between the mother and child is around 25% [164, 165]. However, where combination antiretroviral drug treatment and Cesarean section are available,

this risk can be reduced to as low as <1% [164]. Postnatal transmission through breastfeeding has been demonstrated in a number of studies [166, 167] and can vary from 5% to 20% [168, 169]. Both HIV-1 and HIV-2 can be transmitted from mother to child, but HIV-2 is transmitted much less frequently by the mother-to-child and sexual route than HIV-1 [170, 171]. The sexual transmission of HIV is well documented, the male to female infection after a single exposure to HIV is 0.01-0.32% [172, 173], and the female to male infection after a single exposure is 0.01-0.1% [173]. These estimates are mostly derived from studies in the developed world. In developing countries, several factors like co-infection with other sexually transmitted diseases, poor acceptance of condoms, the numbers of sexual partners, circumcision practices, high viral loads in those infected, can increase the likelihood of heterosexual transmission to 20% or even higher [174].

Infectiousness is especially high during primary HIV infection when virus levels are very high. It has been demonstrated that receptive unprotected anal intercourse with an HIV-positive man is the major behavioral risk factor for HIV transmission among homosexual men [175]. The greatest risk factor for HIV transmission is the high viral load in the infected person who transmit the virus, so reducing the viral load is essential to interrupting transmission [176]. HAART dramatically lowers viral load and numerous studies have demonstrated its potential for prevention of HIV transmission [177-179]. There is also evidence that male circumcision reduces the risk of heterosexually acquired HIV infection in approximately 40-60% [180-182]. Additional evidence on the prevention of sexual transmission of HIV is the use of condoms and reduction in the number of sexual partners [183, 184].

1.4.2 Pathogenesis

The pathogenesis of HIV-1 infection can be divided into three stages; the acute phase, the chronic phase and AIDS (Figure 8). The acute phase, also called primary infection, is characterized by massive viral replication, resulting in high viral levels, around $10^7 – 10^8$ million RNA copies/ml, and loss of CD4+ T-cells. Approximately 50% of patients infected with HIV develop symptoms of acute HIV infection. Over the following weeks the number of CD4+ T-cells in blood start to recover and the viremia is reduced to a semi-steady state level called a setpoint. The setpoint is a good predictor or disease progression [185]. Chronic HIV infection begins after antibodies to the virus have fully developed and the initial immune response is complete. HIV disease with active virus replication usually progresses during this asymptomatic period, and the rate of disease progression correlates with HIV RNA levels. AIDS is the condition that results from long-term (chronic) HIV infection and is defined by an absolute CD4 cell count of less than 200 cells/µL and specific opportunistic infections or malignancies. In the absence of treatment the average time to development of AIDS is approximately 10 years [186, 187]. However, there are some HIV-1 infected

persons who remain asymptomatic for over 10-15 years and they are called long-term non-progressors [186, 188, 189]. In contrast, there are some individuals called rapid progressors who progress to AIDS within two o three years after infection or less [190, 191]. Although HIV-1 and HIV-2 are biologically similar, HIV-2 is less pathogenic than HIV-1 [192-194].

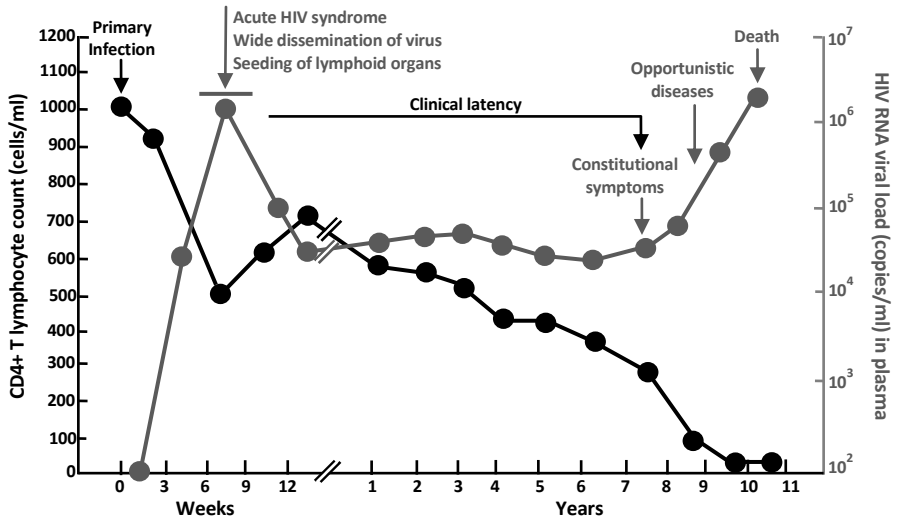

Figure 8. The typical course of the HIV-1 infection. Defined by the CD4+ T-cell counts and the level of viral load.

Disease progression is monitored by observation of clinical symptoms and by quantification of CD4+ T-cell counts and HIV-1 RNA plasma levels. In untreated patients, CD4+ count is the most important laboratory marker, while treated patients are primarily monitored by measuring RNA levels. The plasma HIV-1 RNA level is an important prognostic marker as individuals with high RNA level progress more rapidly to AIDS than those with low levels [185].

1.5 HIV TREATMENT

1.5.1 Antiretroviral therapy

Zidovudine (AZT) was the drug that was first introduced for treatment of HIV infection. AZT is a nucleoside reverse transcriptase inhibitor (NRTI) and was approved by the FDA in 1987. From 1991, additional NRTI drugs became available [195]. In 1995 drugs to a new target, the viral protease, were introduced. In 1996, it was understood that durable and efficient HIV-1 therapy could be achieved by using protease inhibitors in combination with NRTIs. Effective treatment with combinations of antiretrovirals is often referred as highly active antiretroviral

therapy (HAART) or combination antiretroviral therapy (cART). There are currently six classes of antiretroviral drugs: the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), which interfere with HIV-1 replication by competitively inhibiting the reverse transcriptase enzyme, thus leading to chain termination in the synthesis of viral DNA [196, 197]; the non-nucleoside reverse transcriptase inhibitors (NNRTIs) also inhibit the HIV-1 replication by blocking the activity of the reverse transcriptase [197]; 3the protease inhibitors (PIs), which bind to the active site of the protease enzyme, where cleavage of precursor polyproteins occurs, and thereby block the maturation of newly produced viral particles and render them non-infectious [197, 198]; the integrase inhibitors, which block the integrase enzyme that HIV needs to insert its genetic material into human cells. Finally, there are two classes of entry inhibitors, the fusion inhibitors, which bind to the transmembrane domain (gp41) and prevents fusion to the host cell and viral entry [199, 200]; and the CCR5 blockers, which inhibit the entry process by blocking the coreceptor CCR5 [201]. More than 25 antiretroviral drugs have been licensed for the treatment of HIV-1 (Table 2). Additional drugs are undergoing clinical trials (i.e. dolutegravir, elvitegravir, lersivirine, apricitabine, elvucitabine and racivir), and other drugs, like zalcitabine, have been removed.

Like other medications, HIV drugs can cause side effects. Some side effects appear shortly after starting an antiretroviral drug and disappear within a few weeks as the body gets used to the new chemicals. In most cases, the side effects of HIV drugs are mild, like nausea, diarrhea and headache. Unfortunately other side effects are more serious, like liver damage or a severe skin rash. Still other side effects can be: abdominal pain, alopecia, anemia, asthenia, Fanconi syndrome, gynecomastia, hypercholesterolemia, hyperpigmentation, insomnia, lipodystrophy, liver failure, mental confusion, migraines, mitochondrial toxicity, neutropenia, nightmares, oral ulcers, and pancreatitis.

Adherence to HIV treatment is essential to avoid drug resistance. Side effect may contribute to poor adherence. Another factor that may affect adherence is the number of pills taken by the patient. Multi-class combinations products combine two or three antiretroviral drugs into a single pill to increase the efficacy of treatment and the ease of staying on the prescribed medication. Some examples of multi-class combination product are Atripla (efavirenz + emtricitabine + tenofovir), Complera (emtricitabine + rilpivirine + tenofovir), Truvada (Tenofovir + emtricitabine), and Combivir (lamivudine + zidovudine).

The implementation of HAART and its wide use in high-income countries has had a radical change in the HIV epidemic in these countries, extending thousands of lives and greatly improving the quality of life. Epidemiologically, the HIV epidemic in the affected high-income countries has been transformed from an acute viral infection

with an almost universally fatal outcome into a chronic infectious disease. While the introduction of HAART has benefited persons living with HIV in developed countries, these treatments remain inaccessible to many HIV infected persons in developing countries. The obstacles to access life-saving treatment for HIV infection and related opportunistic infections is due primary to the high cost of drugs.

Antiretroviral treatment is the best options for longlasting viral suppression and, subsequently, for the reduction of morbidity and mortality [202-205]. However, current drugs do not eradicate the HIV-1 infection therefore better access to affordable and well-tolerated drugs are needed. WHO and UNAIDS estimate that at least 14.6 million people were in need of antiretroviral therapy in 2009. Many efforts have been done to increase the number of people receiving antiretroviral therapy, but the coverage is still low. As of the end of 2009, 5.25 million people had access to antiretroviral therapy in low- and middle-income countries.

Table 2. Antiretroviral drugs currently approved by US Food and Drug Association.

Drug Class	Name	Approval year
Nucleoside reverse transcriptase inhibitors (NRTIs)	Abacavir (ABC)	1998
	Didanosine (ddI)	1991
	Emtricitabine (FTC)	2003
	Lamivudine (3TC)	1995
	Stavudine (d4T	1994
	Tenofovir (TDF)	2001
	Zalcitabine (ddC)	1992
	Zidovudine (AZT)	1987
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Delavirdine (DLV)	1997
	Efavirenz (EFV)	1998
	Etravirine (ETR)	2008
	Nevirapine (NVP)	1996
	Rilpivirine	2011
Protease inhibitors (PIs)	Amprenavir (APV)	1999
	(fos)-Amprenavir (fAPV)	2003
	Atazanavir (ATV)	2003
	Daranavir	2006
	Indinavir (IDV)	1996
	Nelfinavir (NFV)	1997
	Ritonavir (RTV)	1996
	Saquinavir (SQV)	1995
	Tipranavir (TPV)	2005
	Lopinavir (LPV)	2000
Fusion inhibitors	Enfuvirtide (T-20)	2003
Entry inhibitors	Maraviroc (MVC)	2007
Integrase inhibitors	Raltegravir (RAL)	2007

1.5.2 Antiretroviral therapy in Honduras and Central America

Access to antiretroviral therapy in Central America varies from country to country, and in a way reflects the economic resources of the country. Most people in these countries depend on the public health systems for health care because few have access to social security systems. In some countries like Belize and Nicaragua there is limited access to antiretroviral therapy through public systems; whereas Costa Rica provides universal access to antiretroviral therapy via social security, which is supported by laws and a relatively well-functioning financial mechanism. Most countries, such as Guatemala, El Salvador, Honduras and Panama are in an intermediate stage of development, offering services to those covered by social security systems. However, most people in need of HIV care and treatment are not covered by social security. According to the WHO/UNAIDS, the antiretroviral coverage in Central American countries is relatively low, between 47% and 67%, with exception of Costa Rica that has a 80% of antiretroviral coverage.

Under the initiative for accelerated access to antiretroviral therapy in Central America and the Caribbean, successful price negotiations have led to substantially reduced prices for antiretroviral therapy. While in recent years, the number of people receiving antiretroviral therapy has increased rapidly with the financial support of The Global Fund to fight AIDS, Tuberculosis and Malaria, the access gap still persists and antiretroviral therapy coverage in Honduras is less than 50%. The first-line drug regimen for adults is zidovudine + lamivudine + efavirenz or stavudine + lamivudine + nevirapine. It should be noted that zidovudine and stavudine is no longer recommended for adult patients in high-income countries due to their unfavorable toxicity profile. The first-line drug regimen for children is zidovudine + lamivudine + efavirenz. With support of The Global Fund, Honduras currently has twenty-three Integral Attention Centers for people living with HIV located in major urban areas, which means a limitation for people living with HIV in rural areas with limited economic resources. In Honduras there is still limited access to monitoring tests (CD4 counts and viral load). The main reason for this is financial, i.e. limited budget and high reagents prices, especially for the viral load tests. This limitation in access to CD4 and viral load testing causes clinical problems, such as delays the initiation of antiretroviral therapy and irrational and delayed changes of therapy in patients with treatment failure (see also paper I).

1.5.3 Drug resistance

The rapid evolution of HIV means that suboptimal therapy easily can lead development of drug resistance, especially if they would be used as single agents, i.e. monotherapy. Resistance may significantly contribute to treatment failure and is therefore considered a serious clinical and public-health problem [206-210]. Several

other factors also contribute to antiretroviral failure, such as poor compliance, drug side-effects, and drug-drug interactions. Even if incomplete adherence usually is the primary reason for treatment failure, resistance often develops as a secondary consequence and further aggravates the situation. Thus, several studies have shown that the prevalence of drug resistance among patients failing treatment is up to 75-85% [211-213][I].

Many mutations that confer resistance to different antiretroviral drugs have been described [210]. The most common NRTI mutations include M184V and the thymidine analog mutations (TAMs) [214]. The M184V mutation confers high-level resistance to lamivudine and emtricitabine. TAMs are involved in conferring resistance to all NRTIs, but especially zidovudine and stavudine. The most common NNRTI mutation in patients failing treatment is K103N; this mutation reduces susceptibility to efavirenz, nevirapine and delavirdine. For some drugs like lamivudine and most of the available NNRTI, a single mutation is sufficient to induce high-level resistance. For other drugs like zidovudine, abacavir and most PIs, high-level resistance requires the accumulation of multiple mutations. [210, 214]. Mutations associated with resistance to fusion inhibitor and integrase inhibitor have also been described [215-219].

1.5.4 Transmitted drug resistance

Nowadays, the use of potent combinations of antiretroviral drugs has contributed to minimizing the development of resistance, but is unable to completely eliminate this risk. It is well known that drug resistance mutations can be transmitted by heterosexual and homosexual intercourse, intravenous drug use and from mother to child [220-225]. If resistant HIV variants are transmitted there are several possible evolutionary pathways, because the drug selective pressure is removed as newly infected persons almost always are not receiving antiretroviral treatment. In the absence of therapy some resistance-associated mutations tend to revert towards wild-type amino acids, especially if these mutations reduce the replicative capacity of the virus (e.g. M184V). However, transmitted drug resistance can persist for years as dominant or minority quasispecies in plasma or in PBMCs [226, 227]; indicating that transmitted drug resistance can have a long-term potential impact on response to antiretroviral therapy. The prevalence of transmitted drug resistance among new diagnosed patients varies greatly between 1 and 25% in different studies from different countries [228-234][II]. Both, the development of drug resistance mutations in chronically treated patients and the transmission of drug resistance mutations in new diagnosed patients have important implications for the successful use and management of therapy and therefore constitute a public health concern.

1.5.5 Resistance testing

There are two principal ways to measure HIV-1 antiretroviral drug resistance in routine clinical practice [235]. Phenotypic testing is the most direct method to measure the virus susceptibility to antiretroviral drugs. The virus is cultured in the presence of increasing concentrations of the drug of interest to determine the concentration required to inhibit replication by 50 % or 90 % (IC_{50} and IC_{90}). The recombinant phenotypic assay is a development of the phenotypic assays, in which the pol gene of a patient's virus is amplified from plasma and inserted into an incomplete HIV plasmid to reconstruct an infectious virus, which is tested for drug susceptibility. Genotypic resistance assays detect mutations in the viral genome that are associated with drug resistance by DNA sequencing or equivalent methods. The viral sequence is derived directly from virion RNA in plasma by RT-PCR. The drug susceptibility of the virus is predicted by interpretation algorithms that are freely available online, e.g. Stanford, ANRS, and Rega. A third approach, the virtual phenotyping assay, is a combination of genotyping and phenotyping assays. In this assay, the combination of resistance-associated mutations found by genotyping is matched to a large database of virus variants with both genotype and phenotype. However, virtual phenotyping has not gained wide international use.

It has been shown that phenotypic and genotypic resistance assays provide comparable clinical results, but they still have their individual advantages and limitations. Phenotypic resistance assays provide quantitative data of resistance levels to the different drugs that are tested, but are more expensive and time-consuming to perform. Genotypic assays are by far the most commonly used in clinical practice because they are considerably faster, have lower cost and are more widely available. In conclusion, it is important to measure HIV-1 drug resistance because loss of HIV-1 drug susceptibility is associated with a significant risk of treatment failure. The International AIDS Society-USA panel, the European guidelines, and other expert groups recommend resistance testing in untreated patients with established infection prior to initiating therapy, in patients with treatment failure and during pregnancy [236, 237]. The World Health Organization also recommends resistance testing for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment [238].

2 AIMS

The general aim of this thesis was to investigate the prevalence of drug resistance in Honduras and the molecular epidemiology of HIV-1 in Honduras the rest of the Central American region.

The specifics aims were:

Paper I: To investigate the prevalence of drug resistance among Honduran HIV-1 infected patients who were failing therapy.

Paper II: To estimate the prevalence of transmitted drug resistance among Honduran treatment-naïve, newly-diagnosed HIV-1 infected patients.

Paper III: To investigate when and how HIV-1 has entered Honduras and how variants have circulated in different transmission groups and regions of the country.

Paper IV: To investigate the genetic diversity and molecular epidemiology of HIV-1 in Central America.

3 PATIENTS AND METHODS

3.1 STUDY POPULATIONS

Paper I

The study population consisted of 138 treated HIV-positive Honduran patients with signs of treatment failure and was divided into 97 adults and 41 children under 18 years of age. The patients were recruited from the two major medical facilities in the country, Instituto Nacional del Tórax in Tegucigalpa and Hospital Mario Catarino Rivas in San Pedro Sula. The main selection criterion was a clinical need for HIV-1 resistance testing due to suspected or documented treatment failure. The study samples were collected between June 2004 and April 2007.

Paper II

A total of 230 treatment naïve patients with newly diagnosed HIV-1 infection were included in the study using a published sampling strategy that was designed to obtain a representative sample of HIV-1 infected patients in Honduras [239]. Thus, samples were obtained from different population groups (general population, Garifunas ethnic group, female sex workers and men who have sex with men) and different geographic regions. Samples from the general population were collected using a convenience sampling strategy in the three major clinical centers of the country, Centro de Salud Miguel Paz Barahona in San Pedro Sula, Hospital Escuela and Instituto Nacional del Tórax in Tegucigalpa. Samples from Garifunas were collected using a multistage probabilistic sampling [17]. Furthermore, samples from MSM and samples from FSW were collected using a respondent driven sampling [240]. Samples were collected during April 2004 and April 2007. A total of 200 samples were successfully sequenced.

Paper III

To study the molecular epidemiology of HIV-1 in Honduras we used the same published stratified cluster sampling strategy as in paper II. For this purpose we included 185 patients from paper II as well as 27 patients from paper I. Forty-five additional patients were included. Thus, HIV-1 pol gene sequences from a total of 257 patients were investigated. The study included samples from the general population from central/southern cities (n=94), from northern cities (n=44), and from rural areas (n=26), Garifunas (n=34), FSW (n=27), and MSM (n=32).

Paper IV

This study included 297 newly generated sequences from HIV-1-positive patients from Belize, Costa Rica, El Salvador, Honduras, Nicaragua and Panama. We also included 319 sequences from HIV-1-positive patients from Honduras that were included in

Paper I and Paper II. Finally we included 11 published sequences from Belize [241], and five sequences Panama [242]. Samples from general population in Honduras (n=324), Costa Rica (n=54), Nicaragua (n=52), Panama (n=39) and Belize (n=37) were collected using a convenience sampling strategy in the major clinical centers of the countries and a multistage probabilistic sampling. Samples from MSM and FSW from El Salvador (n=98 and n=48, respectively) and Honduras (n=45 and 40, respectively) were collected using respondent driven sampling. Samples were collected during 2002 – 2010.

3.2 METHODOLOGIES

Analysis	Protocols or Methods	Paper
PBMC DNA and plasma/serum RNA extraction	QIAmp DNA or RNA kit (QIAGEN, Hilden, Germany)	I – IV
Dried blood spots-DNA extraction from Whatman FTA cards	Whatman FTA Purification Reagent (Whatman International Ltd. UK) [241]	IV
Dried blood spots-total nucleic acids extraction from Whatman 903 cards	Modified NucliSens silicon-based extraction (bioMerieux, Inc., Durham, NC) [243]	IV
Amplification of HIV-1 partial *pol* gene	In-house published method [243, 244] [I]	I – IV
Population sequencing analysis	In-house published method, using ABI Prism™ 3100 Genetic Analyzer (Applied Biosystem) [243, 244] [I]	I – IV
Subtype determination	- Phylogenetic tree analysis including HIV-1 subtype references from Los Alamos National Lab (www.hiv.lanl.gov) - REGA HIV-1 automated Subtyping tool v2.0	I – IV
Drug resistance mutations for samples collected from ART patients	2007 version of the International AIDS Society-USA (IAS-USA) drug resistance mutation list at Stanford Genotypic Resistance Interpretation Algorithm [245]	I, III, IV
Drug resistance mutations for samples collected from ART-naïve patients	WHO 2009 list of mutations for surveillance of transmitted HIV-1 drug resistance as implemented in the Stanford Calibrated Population Resistance (CPR) tool [246]	II - IV
Phylogenetic analyses	- Neighbor-joining trees using MEGA 4 and 5 - Maximum likehood trees using PhyML and TOPALi [247]	I – IV III, IV
Molecular clock analysis	BEAST software	III, IV
Determination of recent HIV-1 infections	Immunoglobulin G-Capture BED-Enzyme Immunoassay (BED-CEIA; Calypte Biomedical Corp., Lake Oswego, OR, USA) [248, 249]	II

4 RESULTS AND DISCUSSION

4.1 HIV-1 DRUG RESISTANCE IN HONDURAS

Resistance testing is an important tool to test for transmitted resistance prior to start of first-line therapy and manage treatment failure. At the start of this thesis project, Honduran HIV-medical doctors did not have access resistance testing for their patients. We found that it was difficult and unethical to do research on HIV-1 resistance in Honduras without at the same time provide resistance testing for clinical purposes. For this reason the first step in the project was to establish a system for providing clinical resistance testing for Honduran patients in the short-term as well as the long-term. In the first year of the project, treatment failure patients were identified by their medical doctors. Samples were collected in the hospitals where patients received clinical care, and were sent to the laboratory of virology at the National Autonomous University of Honduras (UNAH). Plasma samples and PBMCs were separated and prepare for shipping to the virology laboratory at the Swedish Institute for Infectious Disease and Control in Stockholm, for resistance analysis. An in-house genotypic resistance assay was used to generate HIV-1 *pol* gene sequences, and sequences were used to identify drug resistance associated mutations and to predict drug susceptibility. Drug susceptibility and relevant patient information, such as actual treatment, years on antiretroviral therapy, number of antiretroviral regimens used, number of antiretroviral treatment changes, clinical stage of disease, level of adherence, CD4 counts and viral load, were used by a group of Swedish experts medical doctors to give email recommendations on treatment based on the antiretroviral drugs that were available in Honduras. When the project was initiated the antiretroviral drugs options and accessibility was very limited, for example lopinavir/ritonavir was very expensive and patients could not received this drug without a previous resistance result, which was very difficult to obtain. Today drug sustainability is better in Honduras, but the access to new and potent drugs is still limited.

4.1.1 Resistance in treated patients [Paper I]

Considering the course of action taken during the initial stages of this project, the samples and results obtained and included in paper I represent a concise sample of patients for whom resistance testing was carried out for screening purposes, as a situation analysis. Because there was very little knowledge about antiretroviral resistance among treated patients in Honduras as well as in Central America we perceived that the findings were of scientific interest. Thus, the rationale of paper I was the fact that some treated-experienced adult and pediatric HIV-1 patients in Honduras had shown signs of treatment failure soon after the National HIV/AIDS

treatment program had begun to provide combination antiretroviral therapy in a consistent way. The HIV-medical doctors who were invited to participate in this study contributed a total of 138 patients; 97 adults and 41 children less than 18 years of age. Patient having virological, immunological or clinical signs of treatment failures were included in the study. These criteria were accepted because the access to plasma viral load measurements and CD4 counts were irregular during the study period. The majority of the patients (91.3%) were at clinical stage B and C according to the CDC classification system. Poor and intermediate adherence was reported in 28.3% of the study participants. The study population consisted of 80 patients who had started combination antiretroviral therapy within the National HIV/AIDS treatment program, 60 of these patients (75%) displayed drug resistance mutations after a median time of 2.6 years on treatment. There were 58 patients (42%) who began therapy before the start of the National HIV/AIDS treatment program. This last group had a median time of 6 years on therapy, and 52 of them (90%) showed drug resistance mutations. An important feature of the study patients was that 52% had started with mono- and dual therapy, whereas 48% had started with triple combination therapy. However, almost all patients had had discontinuous treatment and many “irrational” treatment changes. Start of therapy before the national treatment program was positively associated with the prevalence of resistance (p=0.035). Resistance was also strongly correlated to years on therapy (p=0.001). It was documented that 78% of the patients received more than one different antiretroviral treatment regimen, and resistance was positively correlated with the number of treatment changes (p=0.005). The majority of these treatment changes (49%) were made rationally (e.g. due to suspected treatment failure or drug toxicity), 12% of the cases the treatment changes were irrational, and 17% of the changes involved treatment interruption; these last two changes were often due to cost or interrupted drug supplies. Resistance was significantly more prevalent in patients in whom treatment failure had been identified virologically as compared to immunologically (p<0.001) or clinically (p=0.019).

Drug resistance mutations were found in 122 of the 138 patients [81%, (95% CI: 79-91)]; resistance to at least one drug class was found in 11% of the patients, dual class resistance was found in 43% of the patients and 27% of the patients had triple class resistance. Figure 9 shows the prevalence of drug resistance mutations according to drug class. Resistance was more common in the samples from children [98%, (95% CI: 87-99)] than adults [74%, (95% CI: 64-82)] (p=0.011). The most prevalent NRTI and NNRTI mutations were M184V (62%) and K103N (30%). M184V causes high-level (>100-fold) resistance to lamivudine/emtricitabine and emerges rapidly in patients who receive lamivudine monotherapy [250]. It is also the first mutations to develop in patients receiving partially suppressive triple combination therapy including lamivudine [251-253]. K103N is the most clinically important NNRTI resistance mutation. It causes 20- to 50-fold resistance to the most available NNRTIs, which is sufficient to cause virological failure [254, 255]. It was not surprising to find a high

frequency of the K103N mutation because of the common use of NNRTIs in Honduras and the ability of the virus to develop NNRTI resistance mutations during non-suppressive therapy [255]. At least one major PI resistance mutation was observed in 32% of the study subjects. When this study was carried out the access in Honduras to boosted PIs was very limited this can explain the small proportion of patients with PI resistance mutations.

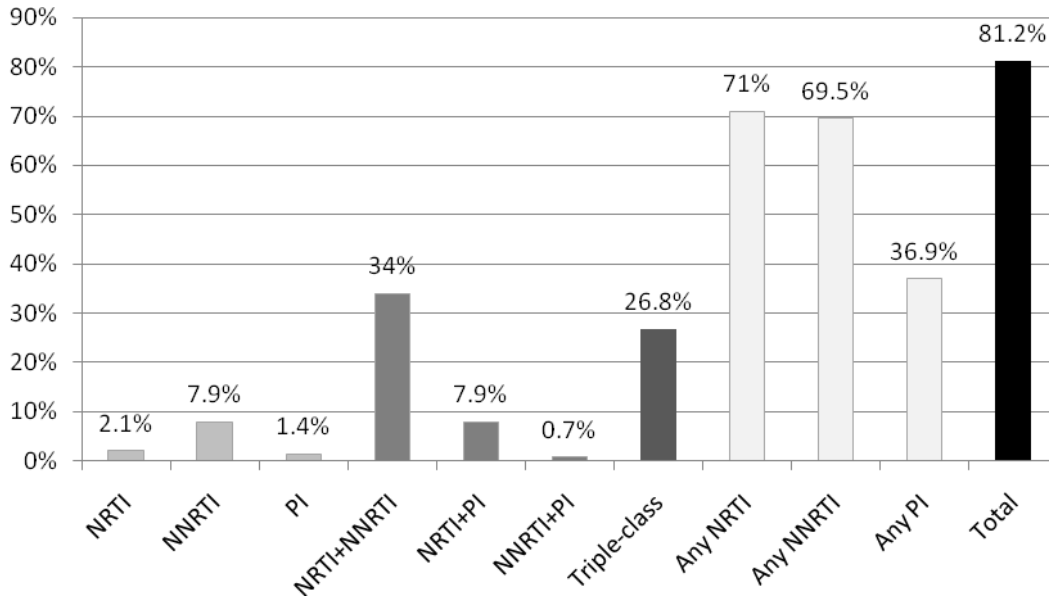

Figure 9. Prevalence of HIV-1 drug resistance mutations according drug-class combination.

The access to CD4 counts and viral load testing was also very limited and the tests were only performed in the national virology laboratory, which serviced all HIV-infected patients of the country. With this project we were able to corroborate the problems faced by the HIV-medical doctors in Honduras with regard to access to CD4 counts and viral load testing for monitoring the course of HIV infection and the response to antiretroviral therapy. Today, CD4 is performed in most of the HIV/AIDS integral attention centers that have laboratories. Viral load is still centralized at the national virology laboratory. Resistance testing is implemented in the laboratory of virology at the UNAH, but is still not available for large scale routine use at the national level. The resistance testing at UNAH is based on our in-house RT-PCR and commercial DNA sequencing abroad. This testing strategy was chosen because it was considered to be more cost-effective and sustainable than kit-based resistance assays or establishment of a sequencing facility in Honduras.

4.1.2 Resistance in newly diagnosed patients [Paper II]

HIV-1 drug resistance can be transmitted and is a current public health challenge. Knowledge about the current patterns of transmitted drug resistance can inform

national planning for HIV/AIDS prevention and care, by anticipating the trends that may affect future ability to effectively treat HIV-positive patients. It can also help HIV medical doctors to select the appropriate and individualized antiretroviral treatment regimens for their patients. WHO and other international guidelines recommend surveillance and testing of drug resistance in newly diagnosed patients. In paper II we evaluated the prevalence of transmitted drug resistance in a representative sample of newly diagnosed HIV-positive untreated patients. We also used a serological incidence assays (the BED assay) to determined whether the patients were likely to have recent or established infections. Partial HIV-1 *pol* sequencing analysis was successful in 200 samples (87%) of the 230 collected study samples. The study group included 132 samples were from the general population, 29 samples from Garifunas, 28 samples from MSM, and 11 samples from FSW. Genotypic resistance testing was done on samples taken within 6 months of diagnosis for all participants; the median time since diagnosis was 6 days (range 1-180 days). Almost all patients were infected by subtype B virus (99%). Two patients (1%) were identified as infected by URFs (unique recombinant forms), URF_AD and URF_AK in the sequenced *pol* gene fragment. According to the BED assay results, 24 patients (12%) were classified as having recent HIV-1 infection and 176 patients (88%) were classified as being associated with established HIV-1 infection. This reflects that many people in Honduras do not know their HIV status and are diagnosed when they already have symptoms indicating that late HIV diagnosis is common in Honduras. This also facilitates the transmission of the virus. Our data indicate that more effort should be done to increase the voluntary testing of HIV in individuals with high risk behaviors.

Transmitted drug resistance was observed in 14 of the 200 study participants, representing an overall 7% prevalence of transmitted drug resistance (95% CI: 3.9%-11.5%). Transmitted drug resistance was found in 18% of the FSW, 14% of the Garifunas, 6% in the general population, and no drug resistance mutations were found in the MSM group. The prevalence of resistance mutations to NNRTIs was 5%, to NRTIs was 3%, and major mutations associated with resistance to PIs were found in only one study participant. A majority of the patients with transmitted drug resistance [64% (9/14)] had only one resistance mutation. To evaluate the possible impact of transmitted drug resistance on the efficacy of future therapy with NRTIs, NNRTIs and PIs, we analyzed the HIV-1 *pol* sequences using the ANRS resistance algorithm. As expected the viruses without signs of transmitted drug resistance [93% (186/200)] were predicted to be fully susceptible to all drugs in the three classes. Among patients with transmitted drug resistance, 12 of 14 patients were predicted to have reduced susceptibility to at least one antiretroviral drug, 71% (10/14) were predicted to have high-level of resistance to the NNRTIs efavirenz and nevirapine. Twenty-nine percent of the patients (4/14) were predicted to have intermediate or high-level resistance to one or several NRTIs. Finally, transmitted drug resistance was identified among 21% (5/24) of specimens classified as recent infection and 5% (9/176) of specimens

classified as established infection. The difference in prevalence of transmitted drug resistance between specimens classified as recent and established HIV infections was statistically significant (p=0.016). The higher prevalence of transmitted drug resistance patients with recent infections could be due an increasing prevalence of transmitted drug resistance over time and/or by reversion of resistance mutations to wild-type in patients with established infections.

Our estimate of the prevalence of transmitted drug resistance in Honduras is similar or slightly lower than the estimates for other studies in Europe, North America, South America, and also slightly lower than that reported by Lloyd *et al.* in a cohort of Honduran patients sampled in 2002 and 2003 at the beginning of the national antiretroviral therapy program [231, 232, 256-259]. We found a moderate level of transmitted drug resistance according to the WHO classification [260]. In countries with moderate level of transmitted drug resistance WHO recommends review antiretroviral therapy programs to investigate potential problems like continuous access to services, drug supplies, drug quality, drug sharing, treatment failures, and to repeat survey in the following year. Honduras has a very well organized HIV/AIDS national program that closely monitors most of issues listed above, but resistance testing is not done in newly diagnosed patients. This year the HIV/AIDS national program of Honduras in collaboration with CDC-GAP for Central America and Panama started a Honduran sexual behavior survey and STI/HIV prevalence survey among vulnerable populations. Within this study all new HIV diagnosed patients will be ask to participate also in an investigation of transmitted drug resistance. Our results have been of great value, but unfortunately still the country is not capable of performing resistance tests in a sustained way, but there are efforts to solve this problem.

4.2 MOLECULAR EPIDEMIOLOGY OF HIV-1

Detailed analyses of the molecular epidemiology of HIV-1, using high-resolution phylogenetics and phylodynamics, can give valuable insights in the origins and spread of the epidemic. In Paper III and Paper IV we provide the first detailed information on the origin and the epidemiological history of HIV-1 in Honduras as well as in five other Central American countries.

4.2.1 In Honduras [Paper III]

Studies in the early 90's indicated that the majority of the HIV strains in Honduran were HIV-1 subtype B [261-263]. More recent studies have showed that HIV-1 subtype B still dominates in Honduras [241, 259] [I, II]. However, there is no data on how many times, when and from where HIV-1 has been introduced into Honduras. Furthermore, there is no information about how HIV-1 subsequently has spread within and between

transmission groups and geographical regions in the country. The objective of paper III was to study the molecular epidemiology of HIV-1 in Honduras using a published stratified cluster sampling strategy, which was designed to obtain a representative sample of the HIV-1 variants that circulate in different transmission groups and regions of Honduras [239]. The study included 257 samples, 164 from the general population, of this group, 94 were from central/southern cities, 44 from northern cities, and 26 from rural areas. We also included 34 samples from Garifunas, 27 from FSW, and 32 samples from MSM. The proportions of females and males were almost the same, 52% and 48%, respectively. The majority of the study participants acquired HIV infection through heterosexual contact (87.5%) and the other 12.5% of the participants were infected through homosexual contact.

Phylogenetic analysis showed that all patients, except two, were infected with subtype B virus. The two non-subtype B sequences were the same URF_AD and URF_AK variants that were described in Paper II. The detailed molecular epidemiology of HIV-1 in Honduras was evaluated using the 255 subtype B sequences. A maximum likelihood (ML) tree was constructed using the 255 Honduran subtype B sequences and 113 carefully selected HIV-1 control sequences (Figure 10). This analysis showed that 221 of the Honduran sequences (87%) formed six monophyletic clusters that were statically supported by approximate likelihood test (aLRT) values >85%. Cluster 1 contained 164 (64%) of the Honduran sequences and two non-Honduran sequences (US and Canada). Thus, this cluster represents the main epidemic in Honduras. The remaining five clusters contained 30, 14, 4, 4, and 5 Honduran sequences, respectively, and no non-Honduran sequences (Figure 10).

The remaining 34 Honduran sequences, which were not included in the six statistically supported clusters, were found in small groups or individually and were intermixed with non-Honduran sequences mainly from the US, Canada, Spain, and Italy. The results indicate that there have been at least six successful introductions of subtype B into Honduras. There have also been other less successful subtype B and non-subtype B introductions. We used high-resolution phylodynamics to estimate the time of the six major HIV-1 introductions in Honduras. The mean time to the most recent common ancestor (tMRCA) for the major introduction (cluster 1) was estimated to 1967, while the mean tMRCAs of the five remaining clusters were estimated to 1966 for cluster 2, 1968 for cluster 3, 1973 for cluster 4, 1984 for cluster 5 and 1984 for cluster 6 (Figure 10). Even though the 95% higher posterior densities (HPDs) of these estimates were relatively broad, our results indicate that HIV-1 was established in Honduras well before the first reports of AIDS in 1981.

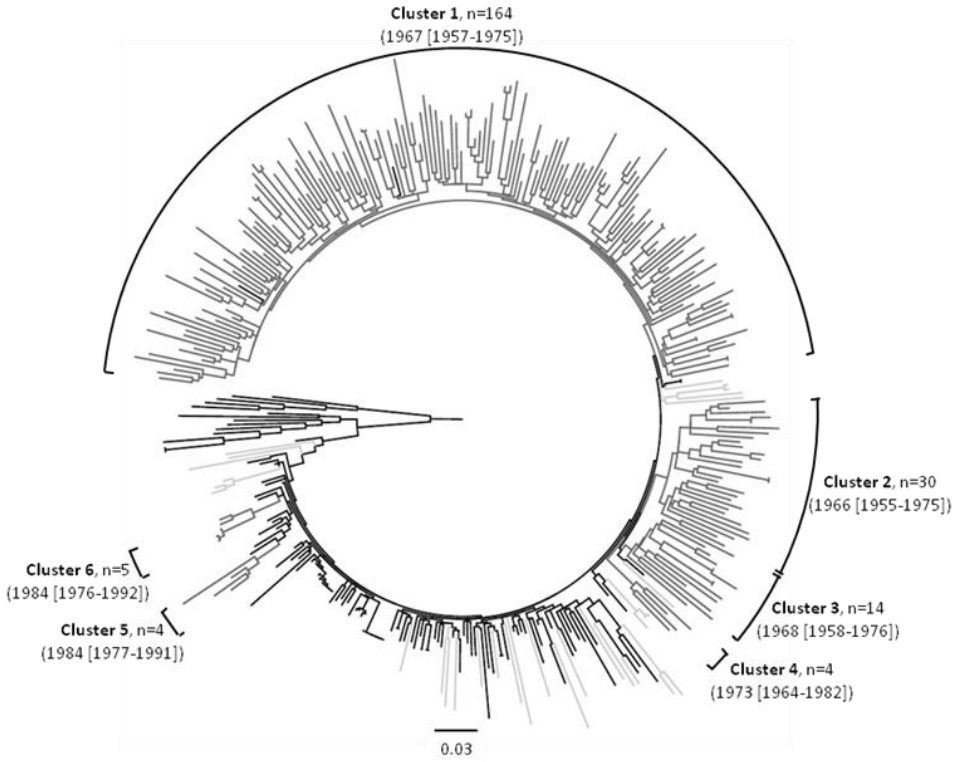

Figure 10. Maximum likelihood tree of 255 Honduran HIV-1 subtype B *pol* sequences. The dataset include sequences from Honduras, closely related database sequences and other reference sequences. The tree was rooted using HIV-1 subtype D. Dark grey branches present the six monophyletic Honduran clusters; light grey branches are non-cluster Honduran sequences, and black branches are non-Honduran sequences. The mean time of the most recent common ancestor of each Honduran cluster is indicated in parenthesis and 95% highest posterior density intervals are indicated in brackets.

It has been suggested that HIV-1 subtype B has spread from Africa to Haiti and from Haiti to the US and the rest of the world resulting in the pandemic clade [33, 162, 264, 265]. However, there is no information on how and when HIV-1 reached Central America and Honduras [266]. To place the Honduran HIV-1 subtype B epidemic into the perspective of the subtype B pandemic, we computed one additional ML tree including all Honduran sequences, all sequences obtained by the BLAST searches, 186 additional US sequences, and all *pol* gene sequences from Haiti available in the Los Alamos HIV database (n=16). The ML tree showed that the majority of the Haitian sequences were positioned close to the root, while sequences from the US and the rest of the world were more distal. Moreover, a majority of the Honduran sequences were even more distally positioned and nested within the pandemic clade. As mention before, it has been shown that HIV-1 subtype B moved from Africa to Haiti around 1962-1970 and then onward to the US and other parts of the world [162]. Taken together, the HIV-1 subtype B epidemiological data and our ML and Bayesian analyses suggest that the successful HIV-1 subtype B introductions into Honduras occurred soon after the epidemic had reached the US in the mid 1960s.

Finally, we investigated the migration of HIV-1 subtype B in Honduras in different geographical areas and transmission groups. For this analysis we divided Honduras into six geographical regions (Figure 11). We found one significant migration from the North (region 2) towards the East (region 3) ($p<0.05$). We also found significantly less migration than expected by chance from Tegucigalpa (region 5) to the North (region 2). These results suggest that HIV-1 subtype B was introduced into the North (mainly in the Department of Atlántida, region 2) from Tegucigalpa (region 5) and then spread locally in the North (region 2), but also continuously from the North (region 2) towards the East (from cities of the Departments of Colon, Gracias a Dios and part of Olancho, region 3). We also observed a number of non-significant migrations from Tegucigalpa to all remaining regions and from region 1 (San Pedro Sula) to all remaining regions, and finally from the North (region 2) to the South (region 6). Apart from the significant migration observed from the North (region 2) towards the East (region 3). However, our results also suggested that nowadays is substantial mixing of HIV-1 subtype B variants within the country. In a similar way we investigated migration between the four main transmission groups; the general population, Garifunas, FSW and MSM. The analyses of migrations between specific transmission groups did not identify any significant migrations ($p>0.05$). However, we identified non-significant migrations from the general population to Garifunas, FSW and MSM and from Garifunas to FSW.

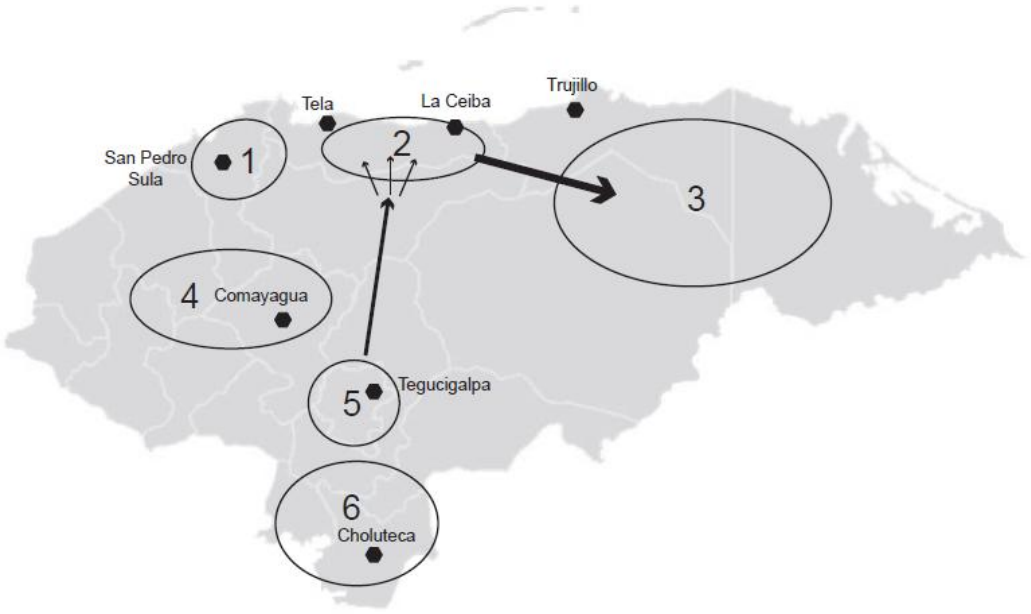

Figure 11. Map of Honduras. The six geographical regions defined are numbered 1-6. The thick arrow indicates genetic flow and the thin arrows indicate an introduction followed by local spread.

Collectively, our results suggest that the two major population centers Tegucigalpa and San Pedro Sula have acted as hubs for the HIV-1 dissemination in Honduras. The first cases of HIV infection in Honduras were reported in the mid 1980's among homo- and bisexual men with a history of traveling abroad, particularly to San Francisco in the US. However, since then the virus has spread primarily in the general population through heterosexual contacts [12]. It has been assumed that the virus introduced into Honduras by MSM who had acquired the infection in the US. However, our results indicate that the virus had been present in Honduras for approximately 15 years before the first cases were diagnosed. This opens a real possibility that the main early introduction may have occurred heterosexually. At least we did observe a clear signal for early viral migration from MSM to heterosexuals. In line with this HIV-1 cases were also reported in the mid 1980's in FSW and in other population groups with a history of having multiple sexual partners. This suggests that the mixing between transmission groups started almost at the beginning of the Honduran HIV-1 epidemic.

4.2.2 In Central America [Paper IV]

The information on the molecular epidemiology of HIV-1 in the Central American region is still very limited. A few country-specific studies in Honduras, Costa Rica and Panama have shown that subtype B is the major HIV variant, although other subtypes, CRFs and URFs have been reported [241, 242, 259, 267] [I-III]. In order to study in more detail the molecular epidemiology of HIV-1 in Central America we obtained 632 *pol* sequences from Belize, Costa Rica, El Salvador, Honduras, Nicaragua and Panama.
We generated 297 new sequences and used 330 published sequences, 325 from Honduras and Belize published by our group [I, II][241] and 5 sequences from Panama published by Ahumada-Ruiz et al. [242]. Almost all sequences (n=625, 98.9%) were of subtype B; five sequences (0.8%) were classified as subtype C, four from Belize and one from Nicaragua; and two sequences (0.3%) from Honduras were identified as URF_AD and URF_AK, respectively [II]. Females and males were almost equally represented in the study population, 49% and 51%, respectively. We included samples from different transmission groups; the predominant transmission route was heterosexual (n=443; 70.6%), 346 of these patients were classified as belonging to the general population, 65 patients were FSW and 32 patients were Garifunas. The remaining patients reported the following routes of transmission: homosexual (114); mother-to-child (67), by blood products (4), and for one patient there was no information.

More than half of the study participants (66%) reported that they never been exposed to antiretroviral therapy, 33.4% were treated patients and in 0.6% of the participants this information was missing. Drug resistance associated mutations were detected in 161 (26%) of the sequences, including 62% of the patients with treatment experience.

In the latter group the prevalence of resistance mutations to any NRTIs was 53%, to any NNRTIs was 49%, to any PIs was 23%, to the combination to at least one NRTI and at least NNRTI was 42%, and to at least one drug from each of the three drug classes (NRTIs + NNRTIs + PIs) was 14%. The most commonly observed mutations were: M184V (46%), T215Y (26%), M41L (23%), K103N (23%), D67N (18%) and K70R (16%).

We found that 7.5% of the treatment naïve patients included in the study were carrying at least one mutation associated with drug resistance. The prevalence of resistance mutations to any NRTIs was 5.1%, to any NNRTIs was 3.6%, to any PIs was 0.2%, and to the combination to at least one NRTI and at least NNRTI was 1.2%. The most commonly observed mutations were: M41L (3.4%), K103N (2.4%), M184V (1.4%), P255H (1%), and T215Y (0.7%). This is the first comprehensive report of transmitted drug resistance in a large group (417) of treatment-naïve patients in Central America. These results have to be interpreted in light of the history of antiretroviral therapy in the countries. In the beginning of the epidemic it was common with uncontrolled mono- and dual therapy with antiviral drugs that were sold illegally or sent from patients' relatives in the US. In more recent years almost all antiretroviral therapy in the Central American countries has been provided through the different national treatment programs. With this as a background the prevalence of transmitted drug resistance is still moderate. I believe that the prevalence may fall in the coming years if countries continue to succeed in having low levels of treatment failure in the national treatment programs.

Detailed phylogenetic analyses were carried out on the 625 subtype B sequences. To identify cross-epidemic relationship that could suggest common geographic origins, three maximum likelihood (ML) trees were constructed with the 625 subtype B sequences from the six study countries, 57 subtype B sequences from neighboring Latin American countries and three sets of random samples of 740 subtype B from the rest of the world. The phyloPart algorithm [268] was used to identify significantly supported clusters including at least 10 isolates [IV]. The phylogenetic analysis showed five statistically supported monophyletic Central American clades (Figure 12). Clade I was largest and included 321 sequences from Honduras, 54 from El Salvador, five from Belize, three from Nicaragua, two from Costa Rica and one from Panama. Despite our efforts to find closely related database sequences using BLAST searches, Clade I was almost exclusively Central American and included only nine non-Central America sequences (four from the US, three from the Bahamas and two from Canada). The four other clusters were smaller. Clade II included 10 strains (five from Belize and five from Honduras); Clade III included 15 Central American sequences (14 from Costa Rica and one from El Salvador); Clade IV included 14 sequences (13 from El Salvador and one from Honduras); and Clade V included 11 Nicaraguan sequences. The fact that the five well-supported Central American clades belonged to separate phylogenetic lineages strongly suggested that they represent independent

introductions of subtype B into Central America. There was no clear pattern of clustering of the Central American clades or other sequences relative to regional or international reference sequences, but many US sequences were proximally located in the tree. An important result is that our BLAST searches did not identify any sequences from Haiti or the rest of the Caribbean as closely related to our Central American sequences. Together with other available epidemiological, phylogenetic, geographical and socioeconomic information this suggests that several of the Central American clades and strains may have originated in the US. This information includes early epidemiological data, as well as other phylogenetic analyses (Gilbert *et al*, 2008), the geographical proximity between Central America and the US, the known tourism between Central America and the US (in both directions), and the legal and illegal immigration from Central America to the US. Finally, the median tMRCA and 95% highest posterior density intervals for the five Central American clades consistently placed the origin of the Central American epidemic between the mid-1960s and the early-1970s (Figure 12).

Finally, I would like to mention that our studies of the molecular epidemiology in Honduras and Central America produced very consistent results. The big Honduran clade overlapped with the big Central American clade. These results indicate that one introduction of HIV into one Central American country, possibly in Honduras, spread to the other Central American countries. This theory can be supported by the social and political situation in Central American countries in the incubation period of the epidemic. In the 1980's Central America was characterized by strong social tension that led to armed conflicts in Guatemala, El Salvador and Nicaragua. With the victory of the Sandinistas in Nicaragua, Central America, and especially Honduras, became the center of the cold war in the Americas. It has been postulated that the strong presence of foreign troops in Honduras was associated with an increase in prostitution and the incidence of STDs in cities where military bases were located [22]. This may have facilitated the spread of HIV in the population. This analysis also support the theory that HIV-1 subtype B was introduced in Honduras and Central America from the US in the early stage of the epidemic. The other smaller clusters that were identified in both analyses show that many other introductions have occurred into the region. The phylogenetic analyses did not show a clear origin of the larger or smaller clusters, but they do not contradict the possibility that many introductions occurred from the US, which seems likely based on epidemiological and socioeconomic information. Nonetheless, our studies have contributed to much a better understanding of origin and spread of HIV-1 in Honduras and the rest of Central America.

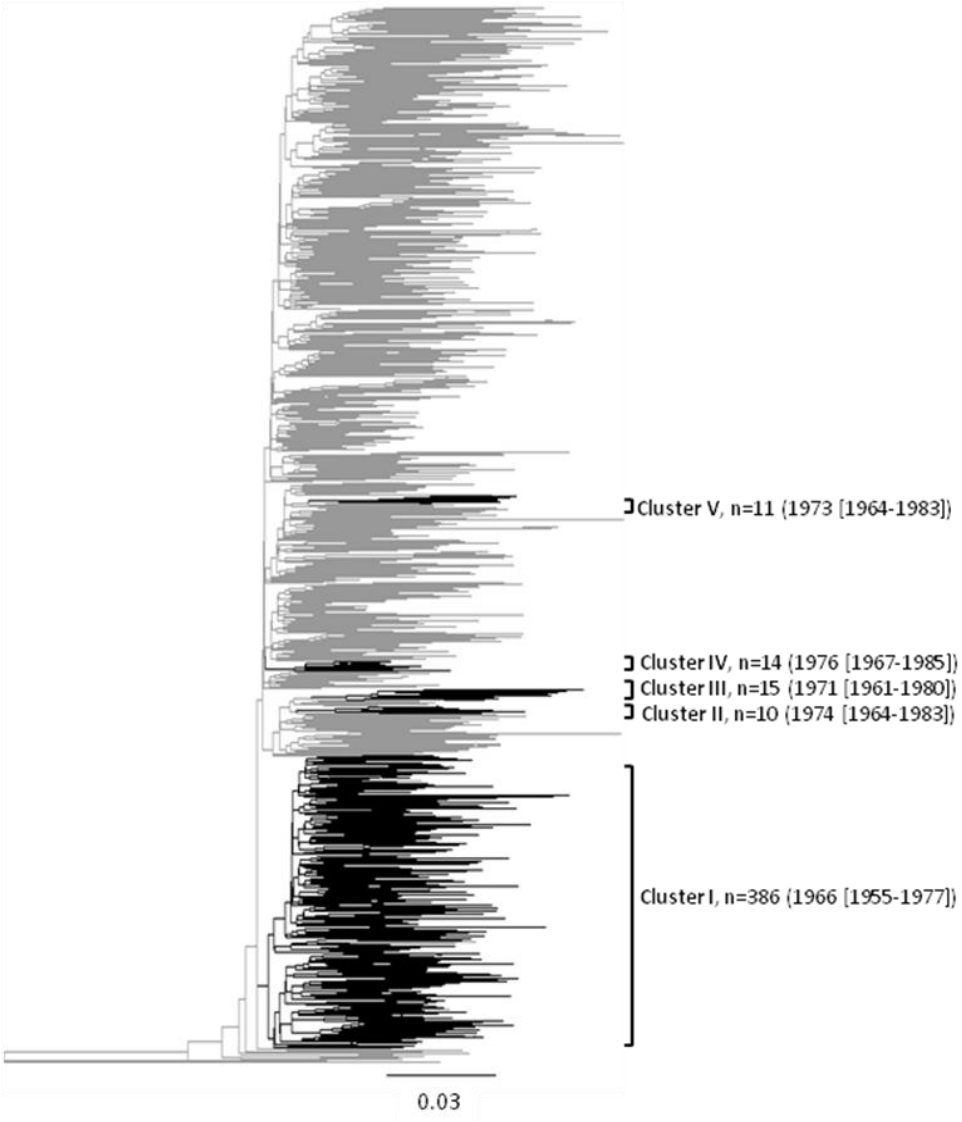

Figure 12. Maximum likelihood tree of the Central America HIV-1 subtype B *pol* sequences. The data set included 625 HIV-1 subtype B strains from the six Central American countries, 57 sequences from Central American neighboring countries and 740 sequences from the rest of the world downloaded from GenBank and the Los Alamos HIV database. The tree was rooted by using HIV-1 subtypes H and C strains as outgroups. The maximum likelihood tree revealed five significantly supported monophyletic clades, formed essentially by Central American sequences (n=436, 70%). The inferred time of the most recent common ancestor of each supported Central American clade is indicated on the right of the label of the clades (dates and 95% highest posterior density intervals).

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Genotyping resistance testing has become the most commonly used method to assess the presence of antiretroviral drug resistance in HIV-1 infected persons. Using this test we documented for first time a high prevalence of resistance to antiretroviral drug among treatment-experienced HIV-1-infected adults and children in Honduras. Several factors that increase the risk of antiretroviral resistance were identified, including previous use of mono- and dual therapy, poor adherence that sometimes was related to toxicity, suboptimal treatment because of economical limitations or irregular drug supply in Honduras. The study also revealed that there was irregular access to CD4 and viral load testing, which negatively affected the possibility to correctly identify treatment failure. Thus, treatment failure identified by virus load testing (i.e. virological failure) was the strongest predictor of resistance, suggesting that plasma HIV-1 viral load testing may be clinically beneficial and cost-effective by preventing unnecessary treatment changes. Our study documented a great need in Honduras for better and continuous access to new generation antiretroviral drugs as well as CD4, viral load and resistance testing. Today, the access to CD4 counts and viral load measurements have improved. There have also been improvements in the number of antiretroviral drugs and their availability. However, resistance testing is still not available in Honduras as a routine test.

It is well-established that drug resistant HIV strains can be transmitted from one person to another and that they may negatively affect treatment outcome. The prevalence of HIV-1 transmitted drug resistance varies according to the history of use of antiretroviral drugs in each country. Because of the above, we estimated the prevalence of transmitted drug resistance of HIV-1 in newly diagnosed patients in Honduras, which was previously largely unknown. According with the WHO classification the transmission of resistance-associated mutations in Honduras was moderate (7%). Furthermore, the recency of HIV-1 infections was low, indicating that late HIV diagnosis is common in Honduras. These results suggest that efforts should be made to increase access and use of HIV testing and also indicate that transmitted drug resistance should be surveyed again in accordance with the WHO guidelines. This would allow identification of possible changes over time in transmitted drug resistance in Honduras.

The global molecular epidemiology of HIV is very diverse. Even though some research groups had studied the history of HIV-1 in the Americas [162, 266], there is limited information about how HIV-1 has entered and spread in the Central American region. For this reason we collected samples of six of the seven Central American countries: Belize, Costa Rica, El Salvador, Honduras, Nicaragua and Panama. We did two separate high-resolution phylogenetic analyses, one which specifically addressed the

molecular epidemiology in Honduras and another that included five additional Central American countries. Our two studies gave highly congruent results. We found that subtype B is the dominating subtype in Central America, although sporadic non-subtype B variants were identified. The analyses also revealed that there have been several subtype B introductions in Honduras and Central America, of which some occurred very soon after the emergence of the pandemic subtype B clade, i.e. in the latter half of the 1960's. One independent introduction, which was estimated to have occurred around 1966-1967, has been particularly successful and accounts for most of the current HIV-1 cases in Central America, including Honduras. The main Central American and Honduran clades were shown to belong to the pandemic subtype B clade, rather the Haitian and Caribbean clades, which suggests that they may have been imported from the US soon after the virus had reached North America.

The studies described in this thesis have contributed to a better understanding of drug resistance and the molecular epidemiology of HIV-1 in Honduras and Central America. The findings about the prevalence of drug resistance have impacted on the national HIV/AIDS response in Honduras. As a research groups we have strengthened the collaboration with the physicians that care for HIV-1 patients at the larger hospitals in Honduras. The project has been helpful for the enrolled patients because the resistance profiles were used together with other clinical and laboratory data to provide individualized treatment advice. Within the framework of the studies of the molecular epidemiology in Central America, our research group has consolidated a good collaboration with other Central American institutions like the Pan American Health Organization, Centers for Control Disease and Prevention's Global AIDS Program for Central America and Panama, and the HIV/AIDS National Program in Belize, Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica and Panama. We have also extended the use of molecular epidemiology to understand the spread of HIV-1 in the Central America region. Finally, we have established a fruitful collaboration with CDC in Atlanta, the University of Florida and the WHO Ecuador in South America. The results presented in this thesis have been shared with the authorities of the National HIV/AIDS programs in the participating countries and will inform treatment policies and prevention programs in each country.

The immediate plan is to perform a phylogeographic analysis of the epidemic in Honduras in collaboration with the University of Florida, to continue investigating the prevalence of HIV-1 drug resistance in Central America and to extend the studies of the molecular epidemiology in Central America by collecting more samples in Belize, Nicaragua, and for first time in Guatemala. Furthermore, we have sequenced samples from Ecuador and are about to start the analyses of these data. Thus, this thesis project has forms the basis for future collaborative research in Central America that hopefully would be translated into improved HIV care and prevention. Even though

much has been achieved there are several challenges for the future, the most urgent is the implementation of routine resistance testing in Central America, including training in the laboratory techniques and the clinical interpretation of the results.

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