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ANTIRETROVIRAL DRUG RESISTANT HIV-1 IN WOMEN AND CHILDREN LIVING IN HONDURAS

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ABSTRACT

Antiretroviral therapy (ART) in HIV infected pregnant women contributes to the prevention of HIV transmission to the newborn. However, as ART can also induce HIV drug resistance during suboptimal levels of virological suppression a major concern is the subsequent risk for transmitted drug resistant (TDR) virus to the child. In Honduras and Belize, like in many other countries around the world, mono-therapy was used to prevent mother-to-child transmission of HIV-1 (MTCT) until relatively recently when it was changed to a more effective combination antiretroviral therapy (cART). Prior to this study there was no information about antiretroviral drug resistance in HIV-1 infected women and children in Honduras and Belize, and limited data other from parts of Latin America. The first aim **(Paper I)** was to evaluate the prevalence of drug resistance in HIV-1 infected infants born in Honduras and Belize between 2001 to 2004, before cART was implemented for prevention of MTCT. Genotypic resistance was performed by sequencing of the HIV *pol* region and was successfully in dried blood spots from 66 HIV-1-infected infants (55 from Honduras and 11 from Belize). Mutations associated with antiretroviral drug resistance were detected in sequences from 13% of the Honduran infants and 27% of Belizean infants. Thus the study documented, for first time, the presence of drug resistance in HIV-1 infected Honduran and Belizean infants. Resistance probably was transmitted from the mothers since none of the infants had received antiretroviral drugs as prophylaxis or therapy.

The second aim **(Paper II)** was to evaluate antiretroviral drug resistance in pregnant HIV-1-infected women in Honduras and risk for MTCT subsequent to ART prophylaxis. In addition, we investigated changes in immune activation during pregnancy by evaluating LPS levels. A total of 50 mother-child pairs and 95 HIV-negative pregnant women were enrolled. The presence of antiretroviral drug resistance was monitored in samples drawn during pregnancy and shortly after delivery. Twenty-nine women (58%) were treatment-naïve at study entry and started antiretroviral prophylaxis against MTCT during pregnancy while 21 women were already identified as HIV-1 infected and on ART at study entry. Antiretroviral drug resistance was detected in 20% of the samples obtained from the mothers at baseline; 10% among treatment-naïve patients and 29% among treatment-experienced patients. Furthermore, despite ART prophylaxis 22 of 50 (44%) women were viremic. No MTCT were observed, but still the high prevalence of resistance and viremia indicated that there was a significant risk for MTCT. The LPS levels declined between pregnancy and after delivery in the HIV-1 infected women indicates that pregnancy might influence the LPS levels, a novel finding that merits further investigation. This study demonstrated for the first time a high prevalence of antiretroviral drug resistance and viremia in pregnant Honduran women, which could limit the effectiveness of antiretroviral prophylaxis against MTCT.

Taken together the studies indicate that there is a need for improvements of prevention against MTCT in Belize and Honduras. This includes better access to monitoring of plasma HIV-1 RNA levels and antiretroviral drug resistance testing.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred in the text by their Roman numerals:

I. ***Parham L,*** de Rivera I, Murillo W, Naver L, Largaespada N, Albert J, Karlsson A**. Short Communication: High Prevalence of Drug Resistance in HIV Type 1-Infected Children Born in Honduras and Belize 2001 to 2004.** *AIDS Res Hum Retroviruses* 2011; 27(10): 1055-9.

II. ***Parham L,*** de Rivera I, Buggert M, García O, Albert J, Karlsson A**. HIV-1 drug resistance and microbial translocation in pregnant Honduran women.** *In manuscript*.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired of immune deficiency syndrome
ART	Antiretroviral therapy
cART	Combination antiretroviral therapy
AZT	Zidovudine
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
d4T	Stavudine
EFV	Efavirenz
env	envelope gene
gag	group specific antigen gene
gp160	gp160
gp120	Glycoprotein 120
gp41	Glycoprotein 41
HIV-1	Human immunodeficiency virus type 1
3TC	Lamivudine
LPS	Lipopolysaccharide
LPV/r	Ritonavir boosted lopinavir
MTCT	Mother-to-child transmission
Nef	Negative factor
NFV	Nelfinavir
NNRTI	Nonnucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PCR	Polymerase chain reaction
PI	Protease inhibitor
pol	Polymerase gene
RAL	Raltegravir
Rev	Regulator of virion expression
RNA	Ribonucleic acid
RT	Reverse transcriptase
sdNVP	Single dose nevirapine
Tat	Viral trans-activator protein
TDR	Transmitted drug resistance
T-20	Enfuvirtide
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WHO	World Health Organization

1 EPIDEMIOLOGY OF HIV-1 INFECTION

1.1 GLOBAL SITUATION OF HIV-1

Infections with the human immunodeficiency virus (HIV) have become one of the major health problems in the world. At the end of 2011, it was estimated that 34 million people were living with HIV, around 2.5 million became infected, and 1.7 million died of the disease [1]. HIV-1 is transmitted in human body fluids by three major routes: sexual intercourse [2, 3], direct injection with the virus-contaminated fluids [4, 5], and by vertical transmission [6, 7]. Sexual intercourse constitutes the main infection route, accounting for 80% of the infections worldwide. Heterosexual transmission is the most frequent transmission route and 70% of all infections are acquired through this route [8-10].

Among the total of HIV infected people worldwide at the end of 2011, 23.5 million are residents in Sub-Saharan Africa infected mainly through heterosexual transmission [1]. The worst affected country on this continent is South Africa, which has 5.6 million infected inhabitants [11]. A large number (4 million) of infected people are also living in South-East Asia of which 50% are concentrated in India [12, 13]. In most Asian countries the main transmission routes are through injecting drug use (IDU), female sex workers (FSM), and men who have sex with men (MSM) [1]. In Eastern Europe and Central Asia the number of infections is rising, reaching 1.4 million in 2011, mainly among IDUs, and their sexual partners [12]. After Sub-Saharan Africa the region with highest HIV prevalence is the Caribbean, where the Bahamas is the nation with the highest prevalence of HIV (3%), heterosexual transmission, is considered the main mode of acquisition of HIV [11, 14]. In Latin America around 1.4 million people were estimated to be living with HIV and there were an estimated 83,000 new infections and 67,000 deaths of AIDS [1, 11]. The highest rates of HIV infection in Latin America are found among MSM, but infection in women and certain ethnics groups is rapidly growing [15].

1.2 GLOBAL HIV-1 SITUATION IN WOMEN AND CHILDREN

The HIV epidemic is a heavy toll on women and children worldwide. At the end of 2011 it was estimated that half of all HIV-1 infected individuals are women [16]. Generally the main transmission mode for HIV in women is through heterosexual contacts [17]. Globally, HIV/AIDS is the leading cause of death among women of reproductive age. In regions such as sub-Saharan Africa and the Caribbean, women are more affected by HIV than men. For Asia and Latin America, approximately 35 percent of adults living with HIV and AIDS are women [11].

One of the most devastating effects of the HIV epidemic has been seen in children. According to the 2012 UNAIDS report, at the end of 2011, there were 3.3 million children living with HIV around the world. An estimated 330,000 children became newly infected with HIV. Almost all of these infections occur in low and middle-income countries [1]. Today, over 25 million children under the age of 15 have lost one or both parents by HIV/AIDS; compared to 1990 when fewer than one million children under the age of 15 were orphaned for this reason [18].

1.3 HIV-1 AND AIDS IN HONDURAS AND BELIZE

With an area of about 112,492 km², Honduras is strategically located in the heart of Central America bordering to Nicaragua, El Salvador and Guatemala Caribbean Sea and Pacific Ocean. Honduras has a population of 8,143,564 as of July

2011. The first case of AIDS in Honduras was reported in 1984 [19]. In April 2012 it was estimated that 30,334 people were infected with HIV-1 of which 21,738 had developed AIDS. Approximately 47% of the HIV infections are in women and 7% in children under 15 years of age. This gives a prevalence of HIV-1 of around 0.8% [0.4–1.4%] [20]. Most Honduran HIV-1-infections have been through the heterosexual route and in the northern region of the country [21]. The highest prevalence of HIV has been observed among FSM (5%), MSM (10%), and the Garífuna population (4.5%) [22, 23]. Garifuna's are descendents from black slaves from Africa who have maintained their culture for over two hundred years. Widespread poverty, poor access to health care, low level of education, low income, lack of employment and migrant labor have been identified as key factors influencing the risk of HIV in the Honduran population. Popularity of traditional myths about HIV is another factor associated to the risk of HIV infection in Garífuna's [24].

Belize is located in the north coast of Central America and has a population of 327,719 inhabitants, in an area of 22,966 km^2. Belize is the only Central American country without a coastline towards the Pacific Ocean and with English as official language. Belize is the country that is most severely affected by HIV/AIDS in the Central American region [25]. The first case of HIV infection in Belize was documented in 1986 [26]. The estimated adult HIV-1 prevalence was estimated to be around 2.3% [2.0 - 2.8%] in 2009. For year 2011, a total of 226 new infections were registered; a decrease of around 7% compared with year 2010. Factors such as multiple partners and early sexual debut are may have influenced the high prevalence of HIV-1 in Belize [25, 27].

1.4 HIV-1 DISCOVERY

In 1981 AIDS was first described as a clinical entity following reports by physicians in the United States of America about young MSM who were suffering opportunistic infections (*i.e. Pneumocystis* pneumonia) and malignancies (*i.e*. Kaposi's sarcoma) along with immunodeficiency [28-30]. In 1983, the virus was isolated and identified from the lymph node of a patient by researchers at the Institute Pasteur in Paris (Barré-Sinoussi et al., 1983) [31]. In the meantime, several reports of similar clinical conditions were coming from different countries around the world [32, 33]. In 1984, Gallo et al demonstrated that the identified agent is the cause of AIDS [34]. In 1986 the isolated virus was officially named *human immunodeficiency virus (HIV)* [35].

1.5 ORIGINS OF HIV-1

Some subspecies of the chimpanzee (*Pan troglodytes troglodytes and P.t.schweinfurthii),* in West Central Africa and western gorillas (*Gorilla gorilla)* have been identified as the natural hosts of the simian immunodeficiency virus (SIV), which were introduced to humans as HIV-1 [36-39]. The most possible transmission route to human beings is through exposure to infected blood or body fluids during hunting [40].

Phylogenetic analysis have shown that *HIV*-1 was introduced to humans through independent transmission events resulting in three *genetic* groups, the group M (major) which was the first introduced around 1910 and is responsible for the pandemic [41]; the group N (non M/non O) and group O (outlier), both seen in West Central Africa. Recently a new group, P, was discovered [42, 43]. The groups M and N have been clustered with SIVcpzPtt strains from Cameroonian chimpanzees [44]. Since group O are more closely related to SIVgor strain it has been suggested that gorillas could be the original reservoir of this strain [38].

2 HIV-1 VIROLOGY

2.1 HIV-1 STRUCTURE

HIV-1 belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae* and *lentivirinae* genus. Each viral particle is spherical with a diameter of 100 - 120 nm and contains two positive-sense single-stranded RNA molecules approximately 10,000 nucleotide long, which are surrounded by nucleocapsid proteins [45]. The RNA contains genes for all HIV-1 proteins including the three major structural genes, *gag, pol and env* (Figure 1). The *gag* gene encodes for proteins that are necessary for the assembly of the viral particle. The *pol* gene encodes for the viral enzymes needed for viral replication; the reverse transcriptase (RT) which transcribes the viral RNA to an intermediate DNA, the integrase (IN) which allow the integration for the viral DNA to the host genome, and the protease (PR) which is involved in the maturation of the virus [46, 47]. The *env* gene, encodes for envelope glycoproteins gp120 and gp41 that are required for viral attachment and fusion to the host cells. The HIV-1 genome also contains genes that encode two regulatory proteins (Tat, Rev) and four accessory proteins (Vif, Vpr, Nef, Vpu) [47]. The main function of the Tat protein is to facilitate the initiation and elongation of the primary transcript. The Rev protein is needed to transport of viral mRNA from the nucleus to cytoplasm. Vif counteracts the cellular antiviral defense protein APOBEC-3G which promotes the infectivity of the virus. Vpr, is a transactivator for host cellular genes and promotes cellular differentiation [48]. Nef inhibits the recognition of HIV infected cells by the host defense system. The Nef protein is expressed at the early phase of HIV infection together with Tat and Rev. Vpu**,** downregulates CD4 on infected cells and thereby enhances the release of the new viral particle [49]. The HIV-1 genes are flanked by two regions called non-coding long terminal repeats (LTR) that are needed for the transcription of the viral genome [45].

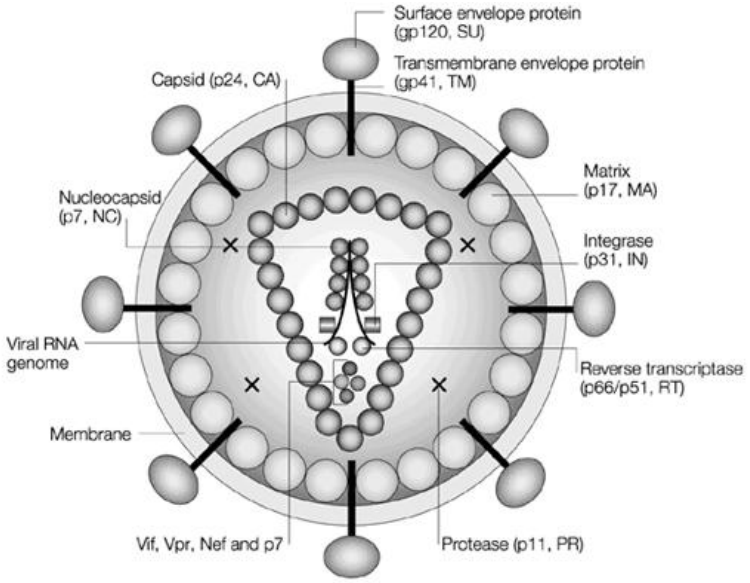

Figure 1. Schematic Structure of HIV-1. Reprinted with permission from [46].

2.2 HIV-1 REPLICATION

HIV begins its life cycle when it binds its gp120 surface proteins to a CD4 receptor that is expressed on the surface of CD4+ T-lymphocytes, macrophages, brain

microglia, and dendritic cell (Figure 2) [50]. After the receptor binding, a conformational change occurs in the gp120 that allows binding to a coreceptor, CCR5 or CXCR4, which triggers viral fusion of the viral envelope with the target cell membrane mediated by gp41. The viral nucleocapsid, which contains the viral genome, it is released into the cytoplasm of the host cell [51].

The reverse transcription process is carried out by the RT enzyme facilitating the synthesis of cDNA from viral RNA. The newly formed preintegration complex containing the viral DNA is transported to the nucleus of the host cell. The viral enzyme integrase mediates integration of the viral DNA into the host chromosome [52]. The integrated viral DNA is called provirus and can remain in a latent stage or become transcriptionally active by the host machinery. The provirus serves as a template for production genomic RNA and messenger RNA which is translated to viral proteins in the cytoplasm. Then, the glycosylated *env* precursor proteins are transported to Golgi apparatus to be inserted at the cell surface [53].

Finally the viral particle is assembled at the cellular membrane to subsequently be released from the cell by budding. It is estimated that the replication cycle of HIV, from entry to viral maturation, has duration of around 2 days. Around 10^{10} new virions are produced daily in an untreated HIV-1-infected patient [54].

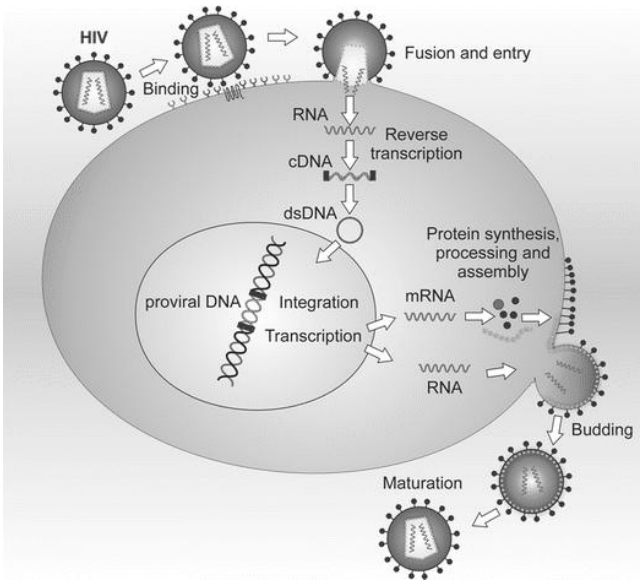

Figure 2. HIV-1 life cycle. Reprinted with permission from [51].

2.3 HIV-1 DIVERSITY

HIV-1 comprises four genetically distinct groups, M, N, O, and the recently identified P [42, 43, 55-59]. The HIV-1 groups have been further classified into subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs), based in phylogenetic analyses of genetic sequences of HIV-1. Some of the factors responsible for the high diversity of HIV-1 are; the lacks of proof reading of the RT enzyme, which leads point mutations during the reverse transcription process (0.1 - 0.3 mutations per genome and replication cycle) [60-62]; and recombination, because the RT makes template switches between the two RNA templates during reverse transcription. If these RNA's are genetically distinct this leads to the formation of a recombinant cDNA product [63, 64].

Group M, which constitutes more than 90% of current HIV infections worldwide, has been divided into nine subtypes, named A, B, C, D, F, G, H, J, and K [10, 65], and more that 50 CRFs (http:www.hiv.lanl.gov). These subtypes have different distribution in the world and a high degree of viral genetic variability (30% in the *env* gene) has been documented for isolates from distinct geographical a [47, 66-68]. Subtype B has been found predominantly in the United States and Europe and several Latin American countries, even if it represents less than 12% of worldwide infections, has been the most studied variant in terms of ARVs susceptibility [69-73]. Subtype C, distributed in Eastern and Southern Africa, India, and parts of China, is the most prevalent subtype in the HIV pandemic, and constitutes half of all known worldwide infections [74, 75]. The previously described subtypes E and I are not longer considered subtypes. The subtype E is now classified as CRFs (CRF01_ A/E) which is highly prevalent in South-East Asia, and subtype I as CRF04_cpx [76-78]. It has been suggested that the high diversity of HIV-1 has implications for transmission, effectiveness of ART and is one of the major challenges for the development of HIV vaccine [79-84].

3 HIV-1 INFECTION

3.1 HIV-1 PATHOGENESIS

HIV-1 infection is characterized by a deterioration of the cellular immune system. The immunodeficiency is characterized by progressive loss of $CD4^+$ T-cells that explains the development of AIDS. The risk of development onset of different opportunistic diseases is correlated with the degree of loss of $CD4^+$ T-cells in blood [85].

Three stages have been identified in the course of HIV infection: acute phase, chronic phase, and AIDS. The acute phase or primary infection lasts a few weeks and is characterized by high viral titers in plasma (10^7 – 10^8 million RNA copies/ml) and a transient depletion of CD4+ T cells [86, 87]. The high-level viremia can be accompanied by flu like symptoms as result of the host immune response [88, 89]. The high virus levels are associated with higher transmission rates per exposure, when compared with later phases of HIV [90]. During the transition to the chronic phase the host immune response partially controls virus replication which leads to a partial recovery of CD4 counts and a decrease of viral load to a level called set point, which is a predictor of disease progression Nevertheless viral replication is constantly active and the high genetic variation of the virus is facilitating emerging immunological escape virus [91-93]. Eventually the infection progresses to AIDS due to the exhaustion of the immune system which is reflected by the depletion of CD4+ T lymphocytes to below 200 cells/ul. At this stage the risk of multiple opportunistic infections or malignancies continuously increases and eventually leads to the death of the patient. The average time from infection to development of AIDS is around 10 years without treatment [91, 94].

3.2 IMMUNE ACTIVATION IN HIV-1 INFECTION

In the first stages of HIV infection, a profound destruction of CD4+ T-cells occurs in mucosal tissues of the gastrointestinal tract [95]. As these cells are not completely replaced, the host remains deficient in memory cells, triggering a lack of control of the microorganisms, which contributes to a more generalized activation of the immune system. Followed the dramatic depletion of CD4+ T cells, the altered integrity of the mucosa cause the leakage of microbial products into the circulation, process known as microbial translocation. This process is evidenced by increased levels of circulating lipopolysaccharides (LPS) in plasma (the major constituent of the cell wall in Gram-negative bacteria) [96, 97].

LPS is a potent immune-stimulator product that activates monocytes and macrophages, which are the major reservoirs of HIV-1 during all stages of infection [98]. Some studies have associated the levels of LPS in plasma to increased immune activation [99-101] which has been proposed as an independent marker of the disease progression [97, 102].

4 ANTIRETROVIRAL THERAPY

Antiretroviral therapy (ART) consists in the use of pharmacologic agents that have specific inhibitory effects on HIV replication cycle. The primary goal of ART is the suppression of viral replication, to promote restoration and/or preservation of immune functions in order to reduce HIV-related morbidity and mortality.

Antiretroviral drugs belong to six classes of drugs which target different steps in the viral life cycle (Figure 3). (1) The nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) are analogues of the naturally occurring building blocks needed to synthesize the viral DNA, and when incorporated into the growing viral DNA chain they cause chain termination [103]. (2) The non-nucleoside reverse transcriptase inhibitors (NNRTIs) interfere with the reverse transcription by directly binding to the enzyme and thereby block the polymerase activity. (3) The protease inhibitors (PIs) inhibit the viral maturation process by binding to the protease enzyme prohibiting cleavage of the viral precursor protein resulting in lack of functional virion formation [104]. (4) The integrase inhibitors target the HIV enzyme integrase, which is responsible for the integration of viral genetic material into the human host cell DNA, a crucial step in the replication cycle of HIV-1 [105]. Viral entry can be blocked by (5) fusion inhibitors and (6) CCR5 co-receptor antagonists that interfere early in the replication cycle with the receptor-mediated entry of the virus into a cell by a blocking process of gp41 domain inhibiting the conformational change of gp41 that is necessary for fusion of virions to the host cell, or blocking the CCR5 co-receptor and thereby inhibiting it interaction with viral surface protein gp120 necessary for viral entry into the host cell [106-108].

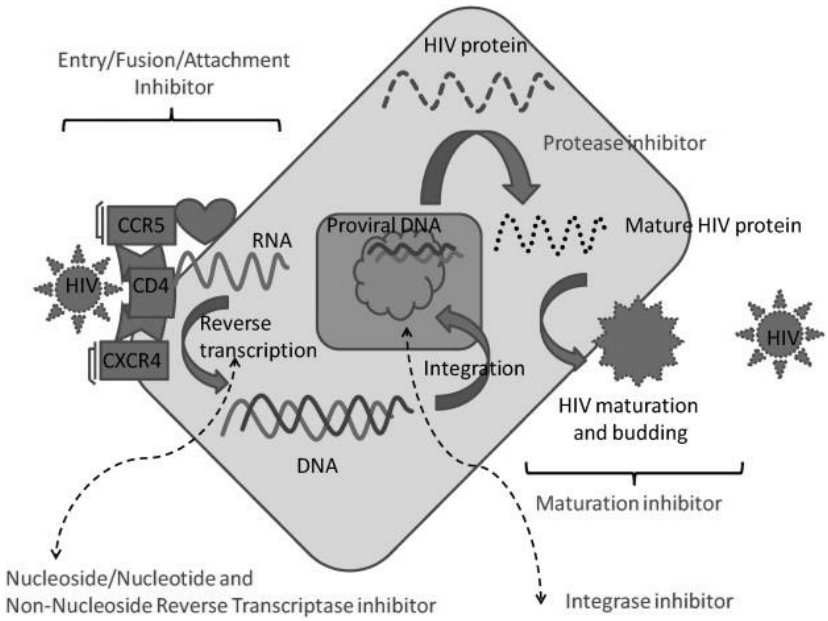

Figure 3. Antiretroviral drug targets for HIV-1 viral cycle steps. Reprinted with permission from [109].

Currently, more than 25 antiretroviral drugs are approved by Food and Drug Administration (FDA-USA) (Table 1) for use in HIV-infected adults and adolescents and 23 have been approved for pediatric treatment; as well multi-class drug combinations. Significant advances have been made through the years to improve the

ART regimens which as improved the quality of life for infected adults and children. The most important advance was the introduction in 1996 of combination antiretroviral therapy (cART), which made it possible to achieve to a maximal and durable virologic suppression. In recent years the main advances has been in terms of reducing the toxicity of the therapy. Nonetheless, the scaling up access in developing countries is still a challenge, mainly in terms of cost. Several important factors have to be considered for the administration of ART including; adverse effects, long-term drug toxicities, complexity of use, adherence and development of HIV-1 drug resistance [110].

Table 1. Antiretroviral drugs approved by FDA for adult treatment infection in adults and pediatric infection.

Class of drug	Antiretroviral drugs for use in Adults	Abbreviation	Year of approval
NRTIs	Zidovudine	AZT (ZDV)*	1987
	Didanosine	ddI*	1991
	Zalcitabine	ddC*	1992
	Stavudine	d4T*	1994
	Lamivudine	3TC*	1995
	Abacavir	ABC*	1998
	Tenofovir	TDF*	2001
	Emtricitabine	FTC*	2003
NNRTIs	Nevirapine	NVP*	1996
	Delavirdine	DLV*	1997
	Efavirenz	EFV*	1998
	Amprenavir	APV	1999
	Etravirine	ETR	2008
	Rilpivirine	-	2011
PIs	Saquinavir	SQV*	1995
	Indinavir	IDV	1996
	Ritonavir	RTV*	1996
	Nelfinavir	NFV*	1997
	Amprenavir	APV*	1999
	Lopinavir	LPV*	2000
	Fosamprenavir	fAPV*	2003
	Atazanavir	ATV*	2003
	Tipranavir	TPV*	2005
	Darunavir	-	2006
Fusion Inhibitors	Enfuvirtide	T-20*	2003
Entry Inhibitors	Maraviroc	MVC*	2007
Integrase inhibitors	Raltegravir	RAL*	2007

*Antiretroviral drugs used in children

5 PREVENTION STRATEGIES OF MOTHER-TO-CHILD TRANSMISSION OF HIV-1

5.1 MOTHER-TO-CHILD TRANSMISSION OF HIV-1

Mother-to-child transmission of HIV-1 (MTCT) is responsible of more than 90% of pediatric infections worldwide [111]. Children can be infected during pregnancy, labor, delivery, and breastfeeding [112].

Without any type of preventive measure, there is a risk of transmission of around 15-30% in non-breastfeeding population. A majority (65%) of these transmissions occur during labor, whereas 35% in utero. An additional risk of 5-20% exists when the child is breastfeed, for an overall transmission rate of 20-45% [113, 114]. Several factors may influence the MTCT such as high maternal viral load levels (mainly in the last months of pregnancy and/or during labor) [115], amount of virus in genital secretions and in the cervix site; low levels of CD4+ T cell count (≤200 CD4+ cell/mm^3) [116, 117]. Another important factor associated to vertical transmission is the duration of membrane rupture, especially if longer than 4 hours [118-120]. Other factors that are associated with the risk of MTCT are co-infections with hepatitis C, as well as breast diseases abscesses and mastitis [121-123]. Viral factors such as certains subtypes and types of co-receptor use have also been associated with an increased risk of vertical transmission [124-127].

The availability of prevention strategies to avoid MTCT of HIV-1 have resulted in a dramatic decline of the infections in this population around the world. Several studies have demonstrated that the risk of MTCT can be substantially reduced if adequate preventive measures are provided. Preventive MTCT interventions include: HIV-1 testing, Caesarian section as mode of delivery before labor and before membrane rupture of, abstaining of maternal breast feeding, and antiretroviral prophylaxis for the mother and the child [117, 128].

5.2 HIV-1 TESTING

One of the key factors involved in the prevention of MTCT is the identification of an ongoing HIV-1 infection in the pregnant women in good time before delivery. The HIV-1 diagnosis enables infected women and infants to benefit from a timely intervention to prevent MTCT. Over the years, important advances has been done in the diagnosis of HIV-1 infections. At the present time, the fourth generation enzyme-linked immunosorbent assays (ELISA) and enhanced rapid tests have been used for screening of adult population including pregnant women [129-131]. Reactive ELISA results are traditionally confirmed by an immunoblot [103], but other testing algorithms based on only ELISAs and rapid tests have proven accurate and cost-effective. Such algorithms have been endorsed by the WHO for use in resource-limited settings [132]. In Honduras, the National guidelines for diagnosis of HIV-1 infection in pregnant women recommends the use of two rapid tests (Determine HIV-1/2 rapid test and OraQuick advance rapid antibody test HIV-1/2).

In addition to the serological tests molecular methods, *i.e.* polymerase chain reaction (PCR), are available for diagnosis of HIV-1-infection. However, these methods are only used for specific issues such as indeterminate results or discordant results between screening and confirmatory results in adults [103].

In children born by HIV-1 infected women, maternal IgG antibodies are transplacentally transferred from week 32 of pregnancy and may remain detectable until the age of 18 months. Therefore antibody detection is generally only used to

diagnose HIV-1 infection in children older than 18 months [133, 134]. HIV-1 infection in younger infants (<18 months of age) is best diagnosed by two separate positive results obtained by PCR detection of HIV-DNA in peripheral blood mononuclear cells and/or HIV-RNA in plasma [103, 135]. Several studies have reported a variation in the sensitivity of PCR testing depending on the infant's age and the type of nucleic acid detected, ranging from 25% to 50% within the first three weeks of age to 96.2% by four to six weeks of age and reaching 100% after seven weeks of age [136, 137]. HIV-RNA assays have shown to be slightly more sensitive than HIV-1 DNA assays among young infants. Nevertheless, because of the reduced amount of detectable HIV-1 RNA in plasma during ART there are concerns about performing RNA assays for diagnosis in infants receiving ART [132, 138]. The use of molecular techniques to detect HIV-1 in infants has also enabled determination of the time of acquisition. If HIV-DNA/RNA is detected within 48 hours of birth it is assumed that the infection was acquired *in utero*, and when detectable first after 7 to 90 days it is considered as intrapartum infection [139].

Currently, the use of DNA-PCR in resource-constrained settings has become a feasible and powerful tool for HIV-1 detection in infants. Importantly, since the introduction and use of filter paper for sample collection it has been possible to overcome the logistical obstacles in the sample collection such as amount of sample, transportation, and storage [138, 140].

5.3 CAESAREAN SECTION

Cesarean section before labor and rupture of membranes, also called elective Caesarean section, is a good strategy to reduce the risk of MTCT by approximately 50% [141]. Several studies have found that elective Caesarean section is an useful intervention among women whom are not under antiretroviral prophylaxis, or only receiving sub-optimal antiretroviral prophylaxis, and for women who have viral load levels greater than 1000 copies/ml [142-144]. In studies done in women who have viral load levels below 1000 copies/ml, no difference have been found in transmission prevalence among vaginal delivery and elective Caesarean section [117, 145, 146]. As some studies have observed an increased risk for postpartum complications among women who undergo Caesarean as compared to vaginal delivery it is important to consider maternal morbidity associated to elective Caesarean section and the risk/benefit ratio when using this prevention strategy [142, 147, 148]. In several, resource-rich countries, including Sweden, Caesarean section is now optional, and not always recommended, for pregnant women with successful cART in the last weeks of pregnancy [149].

5.4 BREASTFEEDING

After the first report of transmission of HIV-1 through breastfeeding in 1985, it has been widely recommended to avoid maternal feeding for children who are born from HIV-1 infected women [150]. However, in resource-constrained regions, where alternative feeding options are not always feasible, breastfeeding is still recommended [151-153]. As the transmission of HIV-1 by breastfeeding in poor settings still occur [154], there are some preventive interventions that have been designed such as the complete avoidance or decreasing the duration of exposure to breastfeeding, decreasing maternal infectivity using chemical or heat treatment to diminish the viral load in breast milk , and use of antiretroviral prophylaxis at time of breastfeeding in women and children [155-158]. Several studies done in sub-Saharan Africa in the context of early cessation of breastfeeding found significant morbidity associated with this type of intervention [159]. About heat treatment of breast milk, it

has been found to lower viral load in the milk without obvious harmful effects, but data are limited [160-163]. Data from several studies have shown that maternal antiretroviral prophylaxis during breastfeeding decreases the risk of MTCT [159].

5.5 ANTIRETROVIRAL TREATMENT PROPHYLAXIS

Treatment and prophylaxis with antiretroviral drugs are the most effective intervention to prevent MTCT and therefore forms the basis of all recommended preventive strategies. In women, antiretroviral prophylaxis reduces viral load in plasma, vaginal secretions and breast milk. The antiretrovirals also cross the placenta and provide the fetus with pre-exposure prophylaxis against transmission of virus in the maternal genital tract and blood at time of delivery [103]. AZT is the antiretroviral drug that has been most widely used in for MTCT prevention, partly because it was the first available antiretroviral drug and also the first drug with demonstrated efficacy in the prevention of perinatal transmission of HIV-1. Thus, the Pediatric AIDS Clinical Trials Group (PACTG 076) in 1994, reported that AZT administered to the pregnant woman between 14 to 34 weeks of pregnancy and to the infant for 6 weeks after delivery reduced the risk of perinatal transmission from 25.5 to 8.3% [164]. Based on these data several intervention strategies using AZT were developed [165]. One was implemented in PETRA trial performed in Africa with a combination of AZT and 3TC started at 36 weeks of pregnancy, intrapartum, and during one week postpartum to the woman and infant, which reduced transmission by approximately 50% compared with AZT alone [166]. Currently AZT is the antiretroviral drug most extensively used in pregnancy and remains an important component of multiple drug regimens using other NRTIs, NNRTIs and PIs.

In 1999, another antiretroviral drug, NVP, which is a potent NNRTI, was used in a single-dose (sdNVP) in the HIVNET 012 trial conducted in Uganda. NVP was administered during labor to women and postpartum to infants and showed a reduction of transmission by nearly 50% compared with intra- and postpartum AZT [167]. Additionally, high effectiveness (72% reduction compared to AZT) was reported by a combination of AZT and sdNVP in the ANRS 1201/1202 DITRAME PLUS A study [168]. However, even if sdNVP is a potent intervention against MTCT it no longer recommended, because it is associated with rapid development of resistance to NVP [169]. Nevertheless, sdNVP is still in used in resource constrained settings if other options are not available.

In more recent years there have been important progress in MTCT prevention by implementation of cART, which have been highly successful in preventing MTCT in developed countries [170]. When successful, cART gives prolonged suppression of viral replication, plasma viral levels below the detection limit (<50 copies/mL), and thereby minimizes the risk of development of drug resistance. Thus, preventive MTCT studies in the US as well as Europe have documented reductions of transmission rates to below to 2% with the use of cART [141, 171, 172]. The World Health Organization (WHO) in its 2010 guidelines for MTCT prevention recommends the initiation of cART prophylaxis as early as 14 weeks of pregnancy. Recommended regimens in this current guideline include AZT + 3TC in combination with one of these drugs: ritonavir boosted lopinavir (LPV/r), ABC or EFV. It is recommended that EFV should be replaced by NVP if the pregnant woman is in the first trimester of gestation [173]. For our project it is also relevant to mention that the 2006 WHO guidelines recommended the use of AZT from 28 weeks of pregnancy plus intrapartum sdNVP + AZT + 3TC; and AZT + 3TC for 7 days postpartum [174]. The prevention of MTCT by the use of antiretroviral drugs also includes the prophylaxis for neonates, by administration of antiretrovirals after birth. The most common antiretroviral drug used

for HIV-exposed infants has been AZT for a period of four to six weeks [173]. Other antiretroviral drugs have been used in combination with AZT for infant prophylaxis, such as NVP, and 3TC [134, 175]. The use of additional antiretroviral drugs for infant prophylaxis mainly depends on maternal history of antiretroviral drug exposure, HIV-RNA levels at or near delivery, premature rupture of membranes, documented maternal resistance to antiretroviral drugs [103, 149].

The use of strategies to avoid MTCT in Honduras began during year 2001, when a pilot project to prevent MTCT based on mono-therapy with AZT or NVP was initiated. In 2003, after considering the prevention of MTCT as a national priority, "The National Program for MTCT Prevention" was implemented to provide better prevention of MTCT throughout the country. Currently, and in relative accordance with WHO guidelines, cART including AZT, 3TC and NVP or more recently AZT, 3TC and LPV/r are used for MTCT prevention in Honduras from 28 weeks of pregnancy [176]. However, currently it is difficult to implement these guidelines for every pregnant woman in Honduras.

6 ANTIRETROVIRAL RESISTANCE

6.1 GENERAL ASPECTS OF ANTIRETROVIRAL RESISTANCE

HIV-1 resistance to antiretroviral drugs is a major cause and consequence of treatment failure among patients receiving ART and is therefore considered a serious clinical problem worldwide.

HIV-1 drug resistance is defined as any change in the nucleotide sequence of the virus that improves viral replication in the presence of an antiretroviral drug [103]. As previously mentioned in section 4, the goal of ART is to inhibit completely viral replication *in vivo* and sustain the effect for as long as possible. However, resistant viral population can evolve in the presence of antiretroviral drugs, especially if they are given in suboptimal concentrations or combinations [177].

Some viral factors promote the development of resistant variants. These factors include: 1) The high turnover of HIV-1, producing around ten million viral particles per day; 2) The high error rate of the HIV-1 RT that results in production of new viral strains with mutations [178, 179]. Other factors that contribute to development of resistance are: 1) Poor adherence to antiretroviral drugs; 2) The level of genetic barrier of the drugs; 3) Potency of the regimen used [180, 181].

There is considerable knowledge about which mutations in the HIV-1 genome mediate resistance to three main drug classes, NRTIs, NNRTIs and PIs [182-184]. The most common mutation to NRTIs is M184V, which emerges rapidly in patients receiving non-suppressive therapy with 3TC or FTC. M184V by itself confers high-level resistance to both antiretroviral drugs. Thymidine analog mutations (TAMs), which are mainly involved in resistance to AZT and d4T, are also common NRTI resistance mutations. For NNRTIs, the most clinically important mutation is K103N, which causes high-level cross resistance to all NNRTIs [182]. In the case of PIs, resistance develops more slowly because high level resistance requires accumulation of several mutations [180]. Resistance mutations to T-20, the only FDA-approved HIV fusion inhibitor, and to RAL, the first approved integrase inhibitor has also been documented [185, 186].

6.2 TRANSMITTED ANTIRETROVIRAL RESISTANCE

The introduction of ART has greatly improvement the morbidity and mortality in HIV infected patients, however, the wide use of ART has led to an increase proportion of drug resistance among patients on therapy. Patients with drug resistant HIV sometimes transmit the infection to others. Such transmitted drug resistance (TDR) is a potential threat to the success of ART [187].

Transmission of drug-resistant viruses occurs irrespective of the route of infection and it prevalence may vary from 1 to 25% in cohorts from different geographical areas and risk groups [188]. Currently the presence of one or more of 93 mutations (34 NRTI mutations, 19 to NNRTIs and 40 PI) associated with drug resistance are considered as evidence of TDR [189].

Several studies have shown that some transmitted resistant strains can gradually revert to wild-type virus due to fitness cost of certain resistance mutations. Other mutations, with lower fitness costs, may be maintained for at least 1–2 years after transmission and also may persist as minority variants [190, 191]. Such minority variants may impact on responses to ART. For example, the NRTI mutation M184V, which causes resistance to 3TC and FTC, reduces fitness and therefore rapidly reverts

after transmission. In contrast, the NNRTI resistance mutations K103N and Y181C have low impact in viral fitness, and therefore revert more slowly [192].

Many studies have reported on TDR in developed countries [193]. However, reports on TDR in resource limited settings are scarcer. This is especially true for TDR among pregnant HIV-1 infected women and in Central America.

6.3 RESISTANCE TESTING

International HIV treatment guidelines recommend that testing of antiretroviral drug resistance should be part of the management and care of people living with HIV. Thus, it has been demonstrated that resistance testing help clinicians to identify which could be the most appropriate antiretroviral regimen for each individual [194-196]. There are two main methods to determine the HIV resistance to antiretroviral drugs: 1) Phenotypic tests and 2) Genotypic tests. In phenotypic resistance tests a patient HIV-1 isolate or recombinant virus with a pol gene derived from the virus of the patient is cultured in presence of dilutions on an inhibitory drug. This test determines the drug concentration that reduces virus replication by 50% (IC50), or 90% (IC90). Then the result is compared with the IC50 or IC90 obtained for a wild-type virus. The genotypic resistance test is based on the analysis of HIV sequences in order to determine the presence of mutations that are known to be associated with reduced sensitivity to antiretrovirals [197, 198]. The interpretation of genotypic test is done by the use of bioinformatics algorithms that are designed to estimate the clinical utility of the antiretroviral drugs based on which resistance mutations are present in the viral strain. The rule-based algorithms ANRS, Stanford HIVdb, and Rega algorithms are the most commonly used [183, 184, 199]. The genotypic resistance test is the most used method to determine the presence of resistance in clinical settings because is considered simpler, faster and less expensive than phenotypic resistance test [184, 200].

6.4 ANTIRETROVIRAL RESISTANCE DURING PREGNANCY

The use of antiretroviral prophylaxis in HIV-1 infected pregnant women has resulted in significant reduction of MTCT [128]. The development of antiretroviral drug resistance in pregnant women is a concern, since it may diminish the efficacy of ART to prevent MTCT and also may limit future options for maternal treatment [201, 202]. When maternal resistant virus is transmitted to the child, treatment options in the infected child may also be limited [203, 204].

Like with other use ART, mono-therapy and dual-therapy in pregnant women is more prone to development of ART. This is especially true if drugs with low genetic barrier, like 3TC and NVP, are used [175, 180, 181]. Regarding to NVP, is well known that its' long half-life increases the risk of development of resistance following cessation of therapy in women who received sdNVP [205, 206]. Thus, sdNVP to pregnant women is associated with rapid development of the K103N mutation. In the HIVNET 012 study NVP resistance was detected in 20% of women who only received a single intrapartum dose. Reversion to wild type virus was seen among these women [207]. Nevertheless, high risk of re-emergence of NVP resistance exists if NVP-based therapy is resumed. The risk of resistance to NVP after administration of two doses in women is higher than the observed after a sdNVP [208]. Also resistance mutations can also arise in pregnant women after HAART-prophylaxis [202, 209].

Resistance to NRTIs and NNRTIs can frequently occur in infants that become infected despite the NVP-based interventions. In the KiBS study done in Kenya, 67% of infants who became infected despite maternal triple prophylaxis had drug resistance, some to both NNRTIs and NRTIs [210]. The SWEN study reported that 92% of infants who became infected had resistance to NNRTIs [211].

With the widespread use of antiretrovirals, TDR is also considered a concern in pregnant women population. Surveillance studies have registered prevalence of TDR higher than 7% in treatment-naïve pregnant women [212-214]. As was previously mentioned the use of antiretrovirals in Honduras for MTCT prevention started around twelve years ago, and today cART is provided to most women who receive antiretroviral prophylaxis against MTCT. However, all pregnant women are not screened for HIV-1-infection and consequently do not benefit from antiretroviral prophylaxis against MTCT. Resistance tests are not routinely used in Honduras and consequently there is very limited information about antiretroviral drug resistance in pregnant Honduran women and HIV-1 infected children born by these women.

7 AIMS

The overall aim of this project was to investigate different aspects of MTCT of HIV-1 in two Central American countries, Honduras and Belize.

The specific aims were:

Paper I: To investigate the prevalence of antiretroviral drug resistance in HIV-1-infected infants from Honduras and Belize born 2001 to 2004.

Paper II: To investigate antiretroviral drug resistance, microbial translocation, and incidence of HIV-1 transmission, following pregnant Honduran women who received antiretroviral prophylaxis to prevent MTCT.

8 STUDY POPULATION AND METHODS

8.1 STUDY POPULATION AND SAMPLE COLLECTION

Paper I

Paper I is based on a retrospective cohort study, which investigated the prevalence of drug resistance in HIV-1-infected Honduran and Belizean infants before cART was recommended for prevention of MTCT in the region. The study was done in collaboration with the National HIV/AIDS Program from Honduras and the Maternal and Child Health Center from the Belizean Ministry of Health. The study included 95 HIV-1 infected infants born between years 2001 and 2004 in Honduras (n=70) and Belize (n=25). Dried blood spots (DBS) were collected from the infants between 2 weeks to 20 months after delivery. Genotypic resistance testing was successful in 66 (70%) of the DBS samples. The lack of amplification in remaining samples could potentially be due to DNA degradation during DBS storage.

Paper II

Paper II is based on a prospective cohort study of HIV-1-infected pregnant Honduran women who received cART as prophylaxis against MTCT. We assessed the risk of transmission to the infants as well as antiretroviral drug resistance and microbial translocation in the mothers. The study was done in collaboration with the Honduran Ministry of Health. A total of 50 Honduran HIV-1-infected pregnant women and their infants were recruited to the study following informed consent. Sample collection was done between November 2007 and September 2010. Twenty-nine of the women were treatment-naïve and 21 were treatment-experienced. The treatment-naïve women were identified as HIV-1-infected when HIV testing was done as part of the prenatal control, while the treatment-experienced women already were patients at the participating health centers when they became pregnant.

Sample collections (Figure 4) were done at three major health centers in the capital city Tegucigalpa: Centro de Salud Alonso Suazo, Hospital Escuela, and Instituto Hondureño de Seguridad Social (IHSS) and two major health centers in San Pedro Sula (SPS): Hospital Mario Catarino Rivas and IHSS-SPS. Plasma samples were collected from the HIV-infected women at two time points: at 20 - 34 weeks of pregnancy (baseline sample) and 3 - 10 days after delivery (follow-up sample). For treatment-naïve women, baseline samples were collected following diagnosis and before the start of antiretroviral prophylaxis. The follow-up samples were collected a few days after delivery, after they stopped antiretroviral prophylaxis. For treatment-experienced women baseline and follow-up samples were obtained during ongoing ART. From the infants DBS were collected at two time points, at 72 hours and 1 month of age, to evaluate their HIV status by HIV-1 DNA-PCR.

An additional objective for paper II was to evaluate microbial translocation during and after pregnancy, by measuring lipopolysaccharide (LPS) in plasma. Microbial translocation has been suggested to drive inflammatory events in HIV-1 infected individuals, and the plasma levels of LPS to be associated with increased levels of immune activation. The levels of LPS were measured at the two time points in samples from 20 HIV-1 infected women who were selected randomly from the study cohort. The LPS levels in the study women were compared with LPS levels in 95 HIV uninfected pregnant Honduran women who were sampled once during pregnancy.

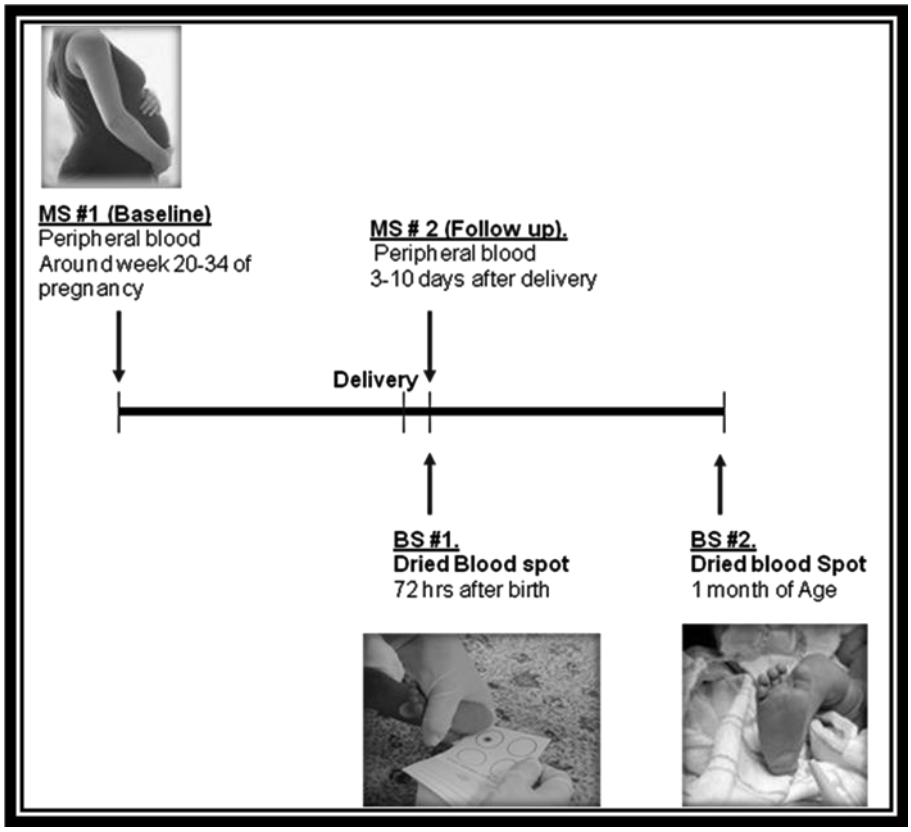

Figure 4. Sample collection for resistance-study among pregnant women and their infants during years 2007 to 2010 (Paper II). MS: sample from the mother; BS: sample from the infant.

8.2 LABORATORY METHODOLOGIES

The Laboratory methodologies used for Paper I and II are summarized in Table 2.

Table 2. Laboratory methods used for studies done in mother-child populations in Paper I and Paper II

Laboratory methodology	Method	Paper
Sample collection from infants	Dried blood spots (DBS). Filter paper from Flinders Technology Associates (FTA cards; Whatman international Ltd. Maidstone, England) [140].	I-II
Sample collection from women	Plasma-preparation tubes (PPT) (Becton Dickinson, USA) Storage at -80°C.	I
DNA extraction from DBS samples	Whatman FTA Purification reagent (Whatman International Ltd. UK) and Tris-EDTA buffer (SIGMA CHEMICAL CO., St. Louis, USA).	I-II
RNA extraction	QIAmp RNA mini kit (Qiagen, Hilden, Germany).	I
Determination of HIV status for infants	In-house nested HIV-1 DNA PCR targeting a region of the *pol* gene (amino acids 17 to 237 of the RT (Beck et al protocol, 2001) [140].	I-II
Genotypic resistance testing	Sequencing of HIV-1 pol gene (amino acids 1–99 in the protease and 1–253 in the RT) with a published *in-house* method (Lindstrom and Albert, 2003 protocol), using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Stockholm, Sweden) using Big Dye terminator sequencing kit (Applied Biosystems, Foster City, California, US) [215].	I-II
Sequence edition/alignment	Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA)/BioEdit version 7.0.9.0 [216].	I-II
Identification of resistance associated mutations in treatment-naïve patients	Calibrated Population Resistance Tool (CPR) (version 5.0 beta updated 01/26/10) for surveillance drug resistance mutations (SDRM) available at the Stanford University HIV Drug Resistance Database (http://cpr.stanford.edu/cpr/servlet/CPR) [217].	I-II
Identification of resistance associated mutations in treatment-experienced patients	Agence Nationale de Reserches Sur le SIDA (ANRS) algorithm (July 2009, version 18) [218].	II
Phylogenetic analysis	Neighbor-joining phylogenetic trees using the MEGA 4 software[219].	I-II
Quantification of plasma HIV-1 RNA	Amplicor HIV-1 monitor system (Roche, Rotkreuz, Switzerland).	II
Measurements of levels of gram-negative bacterial endotoxin (LPS) in plasma	*Limulus amebocyte lysate assay* LAL (Lonza Group, Ltd. Switzerland) using E. *coli* endotoxin standards [96].	II

8.3 STATISTICAL ANALYSIS

Statistical analyses were processed with EpiInfo software 6.4 (Center for Disease Control and Prevention, CDC), GraphPad Prism 5.0 software and InfoStat version 1.1.

8.4 ETHICAL CONSIDERATIONS

Studies performed in Paper I and Paper II, were conducted with the approval of Ethics Committees in Honduras and Sweden (Dnr. 21/ 2009).

9 RESULTS AND DISCUSSION

9.1 DRUG RESISTANCE IN PREGNANT HIV-1 WOMEN IN HONDURAS AND THEIR CHILDREN

The use of ART to prevent MTCT significantly improves the life for HIV-infected mothers and their children. However, if the pregnant women are already infected with HIV-1 resistant strains, or if such resistant strains emerge during prophylaxis, this can cause treatment problems in the woman and the offspring if transmitted. Thus, antiretroviral drug resistance in pregnant HIV-1-infected women is a public health concern.

Prevention of MTCT in Honduras, started during year 2001 by the use of mono-therapy (AZT or NVP) within a pilot program coordinated by the Ministry of Health and UNICEF. The pilot program included only health centers in the two major cities in Honduras, the capital Tegucigalpa and the main industrial center San Pedro Sula. The program was gradually expanded and at the end of 2005 covered 21.4% of the health units in the National Health System. In 2005, 267 pregnant women were diagnosed with an HIV-1-infection, which corresponded to a HIV-1 prevalence of 0.57% in this population. Antiretroviral prophylaxis with mono-therapy and dual-therapy was given to 74% of the HIV-1 infected women [220]. In recent years the National Program has scaled up access to provide better national coverage as well as more efficacious antiretroviral prophylaxis based on cART.

Currently the prevalence of HIV in pregnant women in Honduras is around 0.2% [20]. When this project was initiated in 2005, HIV-1 resistance testing was not available in Honduras and consequently there was a lack of information about HIV resistance in the mother-child population. However, it was recognized that HIV drug resistance is one of the major obstacle for effective treatment and prevention of MTCT. This lead to the initiation of the project "*Molecular Epidemiology of HIV-1 in Central American region and Drug Resistant in Honduras";* a scientific cooperation between National Autonomous University of Honduras and the Swedish Institute for Infectious Disease Control and the Karolinska Institutet in Sweden funded by SIDA/SAREC. One of the aims with the project was to assess the prevalence of antiretroviral drug resistance in Honduran HIV-1-infected pregnant women and its potential transmission to their infants.

The first goal of the MTCT study (Paper I), was to retrospectively evaluate the prevalence of drug resistance in samples from HIV positive Honduran infants born during 2001-2004, i.e. at time when the National Program for MTCT Prevention started and antiretroviral prophylaxis still was based on mono- or dual therapy. In addition, collaboration with Ministry of Health in Belize was established. Belize has among the highest frequency of HIV-1 infected individuals in the Central American region (2.3%), but there was very limited information about prevalence of HIV-1 drug resistance. The MTCT program in Belize formally began in 2002 with the exclusive use of sdNVP [25-27].

The second goal of the MTCT study (Paper II) was to evaluate certain aspects of the current MTCT prevention program (e.g. antiretroviral prophylaxis) in Honduras. This included: 1) The frequency of transmission of HIV-1 from mother to child; 2) The effect on maternal virus levels; 3) The prevalence and development of drug resistance in pregnant women; and 4) Changes during pregnancy in LPS levels, a marker of microbial translocation associated with immune activation.

9.1.1 Antiretroviral drug resistance in infants from Honduras and Belize (Paper I)

The 66 infants analyzed in this study represented approximately 70% of all infants diagnosed in Honduras and 52% of infants diagnosed in Belize during 2001 to 2004 (Table 3). Only 18% of the Honduran mothers, but 72% of the Belizean mothers, reported that they had received antiretroviral prophylaxis during pregnancy.

Table 3. Demographics and characteristics of the HIV-1 infected infants

Demographics	Honduras	Belize
HIV-1 infected infants	55	11
Age in weeks [median (range)]	24 (2 - 80)	16 (4 - 44)
Infants born to mothers receiving preventive ART [n (%)]	10 (18)	8 (72)
Most common type of delivery [n (%)]		
Vaginal delivery	26 (47)	9 (82)
Not available	22 (40)	0
Most common type of feeding [n(%)]		
Formula feeding	27/55 (49)	1/11 (91)
Mixed feeding	24/55 (44)	0

Mutations associated with drug resistance were found in 15% of the infants, with 13% (7/55) resistance in the Honduran infants and 27% (3/11) resistance in the Belizean infants. Among Honduran infants with antiretroviral drug resistance, the age range was between 1 to 13 months. For the Honduran and Belizean babies with resistance, 43% and 67% of their mothers had received prophylactic treatment during pregnancy, respectively.

In Honduran infants we documented antiretroviral drug resistance to all three major drug classes (NNRTIs, NRTIs and PIs). Four infants had NNRTI resistance mutations. The dominance of resistance to NNRTIs is in accordance with other studies [221] and is likely related to the wide use of NNRTI drugs in Honduras, especially NVP that was one of the first drugs available. Furthermore, it has been well established that HIV has the ability to develop NNRTI resistance mutations during mono-therapy due to the low genetic barrier [180]. The long half-life of NVP, which can persist in low levels for more that 3 weeks, also promotes selection of resistance mutations [205, 206]. Similarly, due to the exclusive use of NVP as prophylaxis in pregnant women in Belize during the study period 100% of the resistance mutations found in the Belizean infants were associated with resistance to NNRTIs. In Belize, the use of monotherapy to prevent MTCT continued until 2006 when cART was introduced [25]. For the infants all resistance is considered to be TDR since none of the infants received treatment.

Five of the 10 babies infected with antiretroviral drug resistant HIV-1 were born from women who were not treated during pregnancy, and who reported to be antiretroviral drug naïve (4/7 from Honduras and 1/3 from Belize). This indicates that the women themselves had been infected with resistant virus, which was onwards transmitted to the infants. However, it is difficult to completely rule out unreported use of ART. Several different NNRTI mutations were observed among the infants. The Y181C mutation was the most frequently found (n=3), but also K103N and V106AV were observed. All three mutations confer high-level resistance to NVP and EFV and have been reported in HIV-1 infected infants also by others [177, 222, 223]. K103N,

which is the most important NNRTI resistance mutation [224], is commonly found in HIV-1-infected pregnant women who have received NVP [169].

The phylogenetic analysis shows that 100% of Honduran and 82% of Belizean infants harbored HIV-1 subtype B viral strains. Two infants (18%) from Belize were infected with subtype C, however none of them showed resistance to antiretroviral drugs.

Despite increased access to ART in the Central American countries, there are very few studies investigating the impact of treatment on the level of antiretroviral drug resistance in the region [225-229]. This study (Paper I) was the first conducted to determine the prevalence of MTCT HIV drug resistance in HIV-1 infected infants in Honduras and Belize. The prevalence of TDR found in Honduran infants in this study is higher than the prevalence found in previous studies in adults [225, 226]. Although the sample size was relatively small in both this and earlier studies, it implies that drug resistance is prevalent and needs to be considered already when starting first-line therapy. The study clearly shows that drug resistance and transmitted drug resistance was an important problem among Honduran and Belizean HIV-1-infected pregnant women and their infants when mono-therapy was the only available option to prevent MTCT.

9.1.2 Impact of antiretroviral prophylaxis in HIV-1-infected pregnant Honduran women (Paper II)

The primary objective of this study was to study the prevalence (baseline) and potential development (follow-up) of drug resistance in HIV-1-infected Honduran women receiving antiretroviral prophylaxis during pregnancy (Table 4). The second aim was to evaluate the risk for MTCT. Thirdly, to investigate if pregnancy, viral load, and drug resistance associated mutations had an impact on the level of immune activation by measuring LPS in plasma.

Table 4. Clinical data for the HIV-1 infected pregnant women

	Treatment-naïve women	**Treatment-experienced women**
Patients [n (%)]	29	21
Weeks on prophylaxis [median (range)]	6 (3-20)	-
Years on ART [median (range)]	-	3 (3*-9)
Most common prophylaxis given [n (%)]		
NRTI/PI (AZT + 3TC + LPV/r)	14/16 (88%)	6/9 (66%)
NRTI/NNRTI (AZT + 3TC + NVP)	7/9 (77%)	6/11 (55%)
Plasma virus level at baseline, copies/ml [median (range)]	1,200 (<50 - >100,000)	500 ((<50 - >100,000
Plasma virus level at follow-up, copies/ml [median (range)]	<50 (<50 - >100,000)	<50 (<50 - 44,600)

*Range in Months.

The prevalence of antiretroviral drug resistance in the treatment-naïve women was 14% (4 of 29). Three (10%) had evidence of TDR [187, 189] because mutations were observed at baseline. This prevalence of TDR is comparable with recent findings from a study in treatment-naïve adults in Honduras (7%), which was part of the Swedish-Honduran project [226]. One important fact that has to be highlighted is that the drug resistance associate mutations detected in 3 of the treatment-naïve women

conferred high level resistance to drugs that are commonly used in the country, i.e. 3TC, EFV, NVP [225]. Surprisingly, a high proportion of treatment-naïve women 12 of 29 (41%) had detectable HIV-1 RNA levels in samples collected a few days after delivery despite they were under antiretroviral prophylaxis. Six (21%) of these women had virus levels greater than 10,000 copies/ml. Our results show that the level of TDR and viral loads in treatment-naïve HIV-1 positive pregnant Honduran women is a great concern because it could limit the clinical effectiveness of ARV prophylaxis and indicates that there is a risk for MTCT [149].

The prevalence of antiretroviral drug resistance in the treatment-experienced women was 30% (6 of 21), which is higher than prevalence's reported in other American countries [230-232]. In all of them, the resistance-associated mutations was detected in the first sample collected during pregnancy, meaning, that resistance in those women is likely to be related to ongoing and previous ART. All women had high-level resistance to both NNRTIs available in Honduras, i.e. NVP and EFV [225]. This was primarily due to the presence of the K103N mutation [169]. In addition, three women showed sign of NRTI resistance mutations. The M184V mutation, which is associated with high-level resistance to 3TC, was the most frequently observed NRTI mutation [182, 233]. Both NNRTIs and 3TC have a low genetic barrier towards resistance [180] and as they are widely used in Honduras [225], therefore it is not surprising to find a high frequency of resistance to these drug classes in the country. Thus, the relatively frequent observation of high-level resistance to these drugs in pregnant women is worrisome. Ten of the 21 (48%) treatment-experienced women had detectable HIV-1 RNA levels in samples collected a few days after delivery. Three (14%) had virus levels greater than 10,000 copies/ml, despite receiving ART at the time of baseline sample collection. Moreover, the treatment-experienced women with resistance had significantly higher virus levels at baseline than those without resistance ($p=0.014$; Mann-Whitney U-test).

Collectively the findings in the naïve and treated women indicate that even though no cases of MTCT occurred, the degree of resistance together with the relatively high levels of ongoing viral replication show that there is a significant risk for MTCT in the population [149].

Some studies have found an association between microbial translocation and immune activation [96, 100, 234]. One marker of microbial translocation is the LPS in plasma, which have been measured in different HIV infected populations, but to our knowledge not earlier studied in HIV infected pregnant women. We found that plasma LPS levels were higher during pregnancy, than after delivery in HIV infected women (72 pg/ml, range 23 – 179 pg/ml vs. 46 pg/ml; range 13 – 98 pg/ml; $p<0.001$, Wilcoxon matched pairs test). Interestingly, there was no significant difference in LPS levels between pregnant HIV-positive and HIV-negative women during pregnancy ($p = 0.38$). This could indicate that microbial translocation may naturally increase during pregnancy. Nevertheless as the sample size is too small, further larger studies are needed to confirm our preliminary findings.

In summary, this study shows for first time relatively high levels of antiretroviral drug resistance and ongoing viral replication in the context of antiretroviral prophylaxis and MTCT in Honduras. These findings needs to be considered as an alert indicator of the urgent need to improve the strategies to prevent MTCT in the country including: 1) improvement of antiretroviral prophylaxis regime; 2) availability of genotypic drug resistance tests which at the time is not implemented in the country; 3) better access of viral load test for pregnant women; 4) further studies to determine of rate of MTCT in the country that has been estimated to be around of 0.5%, but the real rate remains unknown.

10 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

One of the most effective strategies to prevent MTCT of HIV is the use of antiretroviral prophylaxis during and after pregnancy. Additionally, ART has been proven to be beneficial to treat pregnant women for their own health [173].

In Honduras, as in other developing countries, there was for a long time use of sub-optimal regimens to prevent MTCT. However, in 2008 this changed by the introduction of cART to prevent MTCT, which is considered the best strategy to minimize HIV transmission [170]. Despite that access to antiretroviral regimens have improved in recent years, very few studies have addressed the prevalence of antiretroviral drug resistance in Central America [225-229]. This includes Honduras where there was no prior information about HIV-1 drug resistance in pregnant women and their infants. As the development of antiretroviral drug resistance remains a major concern worldwide, this project was initiated to study different aspects of antiretroviral prophylaxis and resistance in Honduran pregnant women and infants. Several important results have been obtained in the two papers presented in this thesis. Firstly, we have shown that the prevalence of antiretroviral drug resistance is relatively high in Honduran infants and pregnant women. Secondly, we found that many women were viremic despite antiretroviral prophylaxis against MTCT. Thus, MTCT of both wild-type and resistant strains is an important threat in both Honduras and Belize that requires attention.

Our findings show that it there is a need to improve access and turn-around time of viral load testing for pregnant Honduran women to determine the success or failure of ARV prophylaxis. Furthermore, it would be very valuable if resistance testing could be made available in Honduras in order to provide guidance for preventive treatment and treatment management. This would allow doctors to make timely and informed treatment decision for women with virological treatment failure.

This project has provided new insights into the problem of HIV resistance, treatment, and MTCT that can support the health systems future decisions in relation to improving MTCT prevention strategies. The findings from these studies have raised the alert from the National health authorities and have contributed to strengthen the good collaboration with the physicians and other health personnel, such as nurses and psychologists, at the different hospitals and health centers and also with the National HIV/AIDS Program. This partnership and the relevance of these studies have generated an interest by both sides to continue the close collaboration to seek for grants for future studies in the field of HIV resistance. Through the project "*Molecular Epidemiology of HIV-1 in Central American region and Drug Resistant in Honduras"* a good collaboration with Belize, Guatemala, El Salvador, Nicaragua, Costa Rica, Panama and Ecuador has been established. Moreover, the implementation of new technology and the professional capacities of the personnel that has been generated through the collaboration between Karolinska Institutet and National Autonomous University of Honduras in this study permitted to strengthen the laboratory HIV testing in the country which is going to be beneficial in the future for HIV research in Honduras.

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