Blood Telomere Length in Infants and Their HIV-Infected Mothers Exposed to Antiretroviral Therapy During Pregnancy

by

Tuhina Imam

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Abstract

Background/Objectives:

Nucleoside reverse transcriptase inhibitors (NRTIs) are part of the antiretroviral therapy (ART) given to HIV-infected pregnant women to prevent vertical HIV transmission. The NRTI zidovudine (AZT) is a known inhibitor of human telomerase, the enzyme responsible for telomere elongation. We hypothesized that average telomere length (ATL) may be shorter in infants born to HIV-infected mothers and exposed to ART in utero, compared to ART-unexposed infants.

Methods:

Two independent cohorts of pregnant women and their infants were studied, spanning 1990-2000 (SJ) and 2005-2009 (Pregnancy). SJ included 120 HIV+ exposed and unexposed pregnancies while Pregnancy included 99 HIV+ highly active antiretroviral therapy (HAART)-exposed and HIV- pregnancies. Dried blood spots (SJ) or whole blood (Pregnancy) were collected from the pregnant women and their infants. Relative ATL (rATL) was measured by quantitative PCR. The differences in rATL between HAART/ART-exposed and unexposed maternal, infant and cord blood (CB) were investigated using ANCOVA, adjusting for maternal age, gestational age, smoking (cigarette/marijuana) and illicit drug/methadone use ever in pregnancy. For the HIV/HAART group, additional parameters included CD4+ count, HIV plasma viral load near delivery, length of
HAART exposure, HCV infection and ethnicity. Relationships between maternal and infant rATL were also investigated.

**Results:**

Infant rATL were significantly longer than maternal rATL for both cohorts (p<0.0001). Exposed CB rATL was shorter than controls (p=0.042) but the differences became non-significant after adjusting for covariates. Although a consistent pattern was seen whereby the rATL were 2-6% shorter in the exposed samples compared to the unexposed ones, this difference never reached statistical significance. In the SJ but not the Pregnancy cohort, smoking and illicit drug use in pregnancy were associated with shorter infant (p=0.033) and maternal (p=0.035) blood rATL. In the pregnancy cohort, among the HIV+ HAART-exposed, higher CD4+ count (p=0.047) and longer HIV duration (p=0.016) were independently associated with shorter maternal rATL. Maternal and infant rATL were significantly correlated (r=0.43, p=0.002) in the pregnancy but not in the SJ cohort.

**Conclusion:**

These results suggest that, if HIV and/or ART/HAART is a risk for telomere attrition in the context of this study, it is less important than other recognized risks.
Preface

Ethics Review Board Approvals:

1) Name of Research Ethics Board: UBC Children’s and Women’s Research Ethics Board, Vancouver BC.
   Ethics certificate number (UBC C&W number): H04-70540.

2) Name of Research Ethics Board: UBC Children’s and Women’s Research Ethics Board, Vancouver BC.
   Ethics certificate number (UBC C&W number): H07-03136.

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   Ethics certificate number: 2713

4) Name of Research Ethics Board: Clinical Research Ethics Board, Office of Research Services, UBC, Vancouver BC.
   Ethics certificate number: C04-0540
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<tbody>
<tr>
<td>3TC</td>
<td>Lamivudine/2',3'-dideoxy-3'-thiacytidine (chemical name)</td>
</tr>
<tr>
<td>ABC</td>
<td>Abacavir</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ARV</td>
<td>AntiRetroViral</td>
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<tr>
<td>ATL</td>
<td>Average Telomere Length</td>
</tr>
<tr>
<td>ASPG</td>
<td>Accessory Subunit of Polymerase Gamma</td>
</tr>
<tr>
<td>AZT</td>
<td>Zidovudine/formally called azidothymidine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CD4+</td>
<td>Cluster Designation 4 positive lymphocytes</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>d4T</td>
<td>Stavudine/2',3'-didehydro-3'-dioxythymidine</td>
</tr>
<tr>
<td>ddI</td>
<td>Didanosine/formally called dideoxyionosine</td>
</tr>
<tr>
<td>DKC</td>
<td>Dyskeratosis Congenita</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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</tr>
<tr>
<td>Kb</td>
<td>kilobases</td>
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<tr>
<td>LTL</td>
<td>Leukocyte Telomere Length</td>
</tr>
<tr>
<td>MTCT</td>
<td>Mother-To-Child Transmission</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-Nucleoside Reverse Transcriptase Inhibitors</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside Reverse Transcriptase Inhibitors</td>
</tr>
<tr>
<td>NT-RTI</td>
<td>Nucleotide reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>pVL</td>
<td>Plasma Viral Load</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SES</td>
<td>Socioeconomic status</td>
</tr>
<tr>
<td>STELA</td>
<td>Single Telomere Length Analysis</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>TRF</td>
<td>Terminal Restriction Fragment</td>
</tr>
<tr>
<td>TR or TERC</td>
<td>Telomerase RNA</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
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<tr>
<td>UNAIDS</td>
<td>Joint United Nations Programme on HIV/AIDS</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
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Lastly, I would like to thank my parents and my husband for their love and help, which kept me going during the more difficult times.
Dedications

To my family
1 Introduction

1.1 HIV/AIDS

The human immunodeficiency virus (HIV) is a retrovirus and is the causative agent of AIDS [1]. According to the 2009 AIDS Epidemic Update by UNAIDS, the total number of people living with HIV in 2008 was 33.4 million, 20% higher than that in the year 2000, including 15.7 million women, 2.1 million children under the age of 15, and leading to 2 million deaths. With the use of the successful antiretroviral therapy in both developed and developing countries, the wave of HIV-1\(^1\) seems to have stabilized in many regions. However, the rise continues in Eastern Europe and several parts of Asia and this reflects the fact that new HIV infections arise rapidly [2]. According to WHO 2008, it has been estimated that illnesses related to AIDS will remain as a major cause of premature deaths globally in the years to come.

In the past in developed countries, people who were at higher risk of HIV infection were injecting drug users, sex workers, men who have sex with men, prisoners and mobile workers. However, in recent years, the trend is changing as more infections are due to heterosexual contacts. Most infections in developing countries also arise from heterosexual sex. In 2008, there were approximately 430,000 new HIV infections in children who were under the age of 15 and most of these infections are believed to have arisen through mother-to-child transmission \textit{in utero}, during delivery or breastfeeding [2]. The regional overview by UNAIDS reported that Sub-Saharan Africa still contains the highest population of HIV infections, accounting for 67% of HIV infections

\(^1\) There are two HIV characterized, HIV-1 and HIV-2. HIV-2 is less common whereas HIV-1 is responsible for the global AIDS epidemic.
worldwide. This region also accounts for 72% of the AIDS-related deaths worldwide and 91% of new infections among children [2].

1.2 HIV pathogenesis

1.2.1 Structure of HIV virus

HIV viruses are classified as members of the retrovirus subfamily of lentiviruses [3]. They contain a nucleocapsid core which is surrounded by a lipid envelope which forms during the budding of the virus from host cell membranes [4, 5]. The viral core is made up of approximately 2000 molecules of 24kD capsid protein and it contains the viral genome, the viral protease, integrase, reverse transcriptase (RT), and the various factors which are responsible for the initial steps of HIV virus replication [6]. The HIV-1 genome contains two copies of single stranded RNA molecules each 9.2 kb in size [7]. The HIV genome encodes several structural, non-structural and accessory genes that may alter by mutation but are present in all HIV variants [1]:

- **gag**: encodes the nucleocapsid protein.
- **gag-pol polyprotein**: codes for the three viral enzymes: reverse transcriptase, protease and integrase.
- **env (envelope)**: encodes the protein gp160 which is a precursor, giving rise to gp120 in the exterior and gp41 in the transmembrane.
- **Accessory genes** include tat, vpu, vpr, nef, rev, and vif. Tat, rev and vpr are transactivators whereas vif, nef and vpu are other regulators.
1.2.2 Replication of HIV

The first step in the process of HIV-1 infection is receptor-mediated fusion at the plasma membrane. The interaction occurs between cellular factors of the host cell and gp120, the surface subunit of HIV-1. The key receptor for HIV-1 and HIV-2 is CD4 and the major coreceptors are the chemokine receptors CCR5 and CXCR4 [8]. The high affinity binding of gp120 to CD4 causes the viral protein to undergo a change in conformation which allows gp120 to interact with the chemokine receptors [5]. This attachment in turn causes a conformational change in the transmembrane envelope glycoprotein gp41 that is required for fusion of the viral envelope with the cell membrane. This causes the viral core to enter into the cytoplasm of the cell [5, 9, 10]. The sequential steps involved in the entry have become targets for antiretroviral therapy [11].

The preintegration complex contains the genomic RNA, the viral enzymes reverse transcriptase, integrase and protease and several viral proteins that are responsible for transport through the cytoplasm, for entry into the host cell nucleus and for integration of the viral DNA into the host genome [12, 13].

Reverse transcription is performed by the error-prone reverse transcriptase enzyme of HIV and it uses the RNA strand inside the virus to produce a complementary DNA (cDNA). Once inside the nucleus, the viral DNA is integrated into the host cell genome and this is carried out by the integrase protein [14, 15]. Viral transcription by cellular RNA polymerase II starts once the proviral DNA has been incorporated into the host genome. The gag, pol and env genes produce the polyproteins Gag, Gag-Pol and Env. The viral protease cleaves the Gag protein to form the matrix, capsid and nucleocapsid proteins and the env gene encodes the precursor protein gp160 which will
form gp120 and gp41. The Gag-Pol polyprotein contains the viral enzymes RT, protease and integrase. Once translation is completed, all these proteins move towards the cell membrane and insert themselves there [16]. The Vpu protein forms ion pores that helps in the release of the virus from the host cell [17].

Budding stimulates the protease enzyme which cleaves the Gag and Gag-Pol polyproteins in order to release the structural proteins (matrix, capsid and nucleocapsid proteins) and enzymes (RT, integrase, protease) to form the nucleocapsid and reorganize the virions into their characteristic structure [18].

1.2.3 Natural history of HIV infection

HIV infection occurs through either one of the two routes: mucosal or parenteral. Mucosal transmission occurs when the vaginal, rectal or oral mucosa is exposed to the virus during sexual contact. It also refers to transmission through breast-feeding. Parenteral transmission refers to the infection with the virus by other means, usually injection drug use [19]. The target cell for HIV-1 are CD4 T lymphocytes which are progressively damaged during the course of infection [20].

The typical course of HIV infection consists of three stages:

1) Acute or primary phase – Primary infection usually lasts for about 2-8 weeks and is evident by a marked increase in plasma HIV RNA levels which can go over a concentration of $10^7$ copies/mL [21]. This level gradually declines due to CD8+ cytotoxic T lymphocyte responses [22] and it causes the viral levels to reach a steady state called the “set point” which indicates a balance between the generation of the virion particles and their destruction [23]. However, there is a temporary but dramatic decline in the levels of CD4 T lymphocytes at this phase,
but the level returns to normal after 3 to 4 months of the infection [24]. During primary infection, some individuals may show certain clinical symptoms like lymphadenopathy, fever and pharyngitis [25].

2) Asymptomatic phase – The lymphoid organs, including the lymph nodes, spleen, liver and gastrointestinal tract play major roles in the replication and clearance of virions. At this chronic stage of the disease, there is a dynamic increase in viral replication (10^8 virions/day) in these lymphoid organs such that the levels of infected cells are more than in the peripheral blood [26]. The loss of CD4^+ T cells at this stage is due to the increased cell death by rapid replication of the virus. The increased turnover of CD8^+ T-cells reflects HIV-specific cytolytic T-lymphocyte response. Compared to that during primary infection, the amount of CD8 cytolytic cells return to a level above normal and stays raised till the final stage of the infection [24]. This immunologic response causes the “set point” to be reached after 6 months of infection where the HIV-RNA amounts stabilizes to a level that stays constant during this chronic asymptomatic phase [27].

3) Symptomatic phase – The symptoms that define AIDS occur when the CD4 count falls below 200 cells/μl and this is when viral load shows a sharp increase in the compartments of the peripheral blood [19]. The immune system maintains a balance of specific T cells but this homeostasis is lost about 18 months before the onset of AIDS. Total T cells are lost at this point resulting in severe collapse of the immune system [28] and increased susceptibility to opportunistic infections.

The course of HIV infection is very variable among individuals. Without antiretroviral therapy, the time between infection with HIV to the progression of AIDS in some adults is 8 to 10 years; these long-term non-progressors maintain lower viral loads, stable CD4
counts and thus a functional immune system for more than a few years after primary infection with HIV [29]. However, some individuals develop AIDS within 5 years of the initial infection whereas others do not develop the acquired immunodeficiency syndrome for more than 15 years. Several host and viral factors are involved in influencing the development of AIDS [24]. Such factors may include genetic factors, age, other viruses or general lifestyle [30]. For example, in adults, greater age have been shown to lead to faster progression to AIDS due to rapid decline in immune system [31]; hormonal changes during pregnancy influence progression to AIDS [32]; inheritance of specific genes such as the ones which act as receptors for HIV-1 [33] or those which are involved in effective immune responses also act as factors in the development the disease [34].

1.2.4 No cure, no vaccine: A chronic disease

Half of the people worldwide who have been infected with HIV have died since the identification of HIV as the causative agent for AIDS. Several efforts for the development of HIV vaccine have proven unsuccessful. This is due to several challenges that HIV poses, such as its unusual diversity, its ability to escape the immune system and its latency [35]. In the many years of HIV/AIDS investigation, there has been major advances in HIV therapy which has been successful in decreasing the rate of morbidity, improving the quality of life and extending survival [36]. However, reports on drug resistance [37], increasing risks of infection in at-risk populations and inaccessibility of HIV therapy in some developing countries have surpassed the helpful developments. Progress has been made in the treatment of HIV but failure in preventing new infections is a reminder that a cure is yet to be found [35].
1.3 HAART

The advent of highly active antiretroviral therapy (HAART) in 1996/7 has transformed the treatment of HIV infection by improving the quality of the patient’s life, increasing survival, decreasing opportunistic infections, and effectively decreasing the development of resistant viral strains [38]. HAART has also been successful in reducing the risk of vertical transmission to <1% in regions where there are adequate resources [39]. HAART regimen consists of at least three drugs from at least two classes: two nucleoside reverse transcriptase inhibitors (NRTI) and one protease inhibitor or one non-nucleoside reverse transcriptase inhibitor (NNRTI). The focus here will be on these three classes of drugs as they are the main ones recommended for HIV-infected pregnant women. HAART has been successful in the reduction of plasma viral loads to undetectable levels and repairing the function of the immune system by increasing the levels of CD4+ cells [40]. Current treatment guidelines recommend initiating HAART for patients who are asymptomatic with a CD4 count of ≤ 500 cells/μl, or for those who are symptomatic with an AIDS-related illness [41]. There are now 21 FDA-approved antiretroviral (ARV) drugs available in six classes that are active against HIV. These include: 1) Entry inhibitors, 2) Fusion inhibitors, 3) Integrase inhibitors, 4) Protease inhibitors (PIs) 5) NRTIs and the closely-related nucleotide reverse transcriptase inhibitors (NtRTIs) and 6) Non-nucleoside reverse transcriptase inhibitors (NNRTIs). Each of these drug classes play distinct roles in the viral life cycle and exhibit distinct safety profiles and toxicities. However, the first 3 classes listed above are not typically used in first line HAART regimens [38, 42].
1.3.1 Antiretroviral drugs

1.3.1.1 PIs

The target of PIs is the viral protease to which it binds and prevents the viral polypeptide from being cleaved into its constituent proteins [42]. The first line agent of the PI treatment option includes lopinavir (LPV) in combination with ritonavir [43]. Other frequently used PIs are nelfinavir (NFV), atazanavir (ATV) and darunavir (DRV).

1.3.1.2 NRTIs and NtRTIs

NRTIs are the backbone of all HAART regimens and were the first antiretroviral drugs approved for the treatment of HIV infection. Both NRTIs and NtRTIs need to be phosphorylated by cellular kinases in order to be incorporated into the HIV DNA by HIV reverse transcriptase. NtRTIs need to be biphosphorylated whereas NRTIs need to be triphosphorylated [44]. Both these drugs compete against the naturally occurring nucleoside triphosphates in order to bind at the nucleotide-binding site in the catalytic cleft of the reverse transcriptase enzyme. NRTIs and NtRTIs lack the 3’ hydroxyl group of the deoxyribose, therefore they cannot form a phosphodiester bond with the 5’ phosphate group of the incoming nucleotide, leading to DNA chain termination [45, 46].

The frequently used NRTIs include thymidine analogs such as zidovudine (AZT), stavudine (d4T), cytidine analogs such as lamivudine (3TC) and emtricitabine (FTC), guanosine analogs such as abacavir (ABC), and adenosine analogs such as didanosine (ddI). Tenofovir (TDF) is the only NtRTI that is currently used in HAART regimens [38] (Figure 1).
1.3.1.3 NNRTIs

NNRTIs are non-competitive inhibitors of RT and do not undergo activation by cellular enzymes for their function [46]. Unlike NRTIs, they are not incorporated into the viral DNA chain. Instead, they bind to a pocket in the HIV-1 RT near the NRTI binding site [47]. Binding of NNRTIs to the target distorts the active site of the RT polymerase, affecting polymerization of the nascent chain [48]. The four currently used FDA approved NNRTIs are nevirapine (NVP), delavirdine, entavirine (ETV) and efavirenz (EFV). EFV is the preferred choice in the NNRTI class and has been used as the central component in current ARV regimens. As this thesis focuses on HIV pregnancy, other classes of drugs will not be discussed here as they are rarely ever used in pregnancy [41].
Figure 1: Structures of the available nucleoside reverse transcriptase inhibitors (NRTIs) and a nucleotide reverse transcriptase inhibitor (NtRTI).

Because they lack a 3’-OH group on the deoxyribose sugar, they act as chain terminators, inhibiting HIV replication.
1.4 HIV and pregnancy

Women of child-bearing age comprise ~50% of HIV-infected individuals globally and it is estimated that every year at least 3.28 million pregnancies with HIV infection occur [49]. The prevalence of HIV infection in pregnant women varies from less than 1% to greater than 40% depending on where they live, with the highest rates in Sub-Saharan Africa. According to a 2010 press statement by UNAIDS, about 60% of new infections in Sub-Saharan Africa are in women and the prevalence of HIV among women aged 15-24 years is approximately three times greater than in men in the same age range. As mentioned in section 1.1, most new HIV infections occurring in children are believed to have arisen through perinatal transmission [2]. Due to the greater access to care and treatment, maternal mortality rates are lower in developed countries than in developing ones. In developing countries with high HIV prevalence, HIV disease is one of the major causes of maternal mortality. For example, in Malawi, there were 1221 deaths/100,000 live births in 2002 with AIDS as the leading cause [50]. A study in Zambia showed that there was an 8-fold increase in maternal deaths in the last 2 decades with AIDS-related tuberculosis being one of the main causes of death [51]. However, the introduction of HAART has allowed the decline of maternal mortality and AIDS-associated complications in pregnancy [52].

Whether immune system alteration during pregnancy has any effect on the progression of HIV infection has been a topic of concern. Recent studies have confirmed that pregnancy does not have any adverse effects on the course of HIV infection or survival [53]. However, infection with HIV has been shown to cause some adverse effects on pregnancy [54].
Opportunistic infections that occur during the course of HIV infection complicate pregnancy [55]. Since women of reproductive age are the fastest growing HIV-infected population, early diagnosis through testing, prophylactic antiretroviral therapy, and refraining from breast-feeding are all recommended in order to reduce the risk of vertical transmission [56].

1.4.1 Mother-to-child transmission of HIV

Mother-to-child transmission (MTCT), also called vertical transmission, can occur in several ways: in utero, during labour and delivery (intrapartum), or postnatally (during breastfeeding) [57]. Most MTCT occur during delivery [58]. The rate of vertical transmission varies widely, ranging from less than 2% in developed countries to more than 30% in the developing ones. This is due to the fact that the developed world has easy access to antiretroviral therapy and other interventions to prevent MTCT whereas access to therapy is limited in the developing world [59]. Several studies have shown that the risk of MTCT is affected by exposure of the fetus to infectious maternal body fluids during labour and delivery, prematurity and breast-feeding [49]. There are several maternal, obstetrical and neonatal factors which pose as risks that augment the chance of perinatal transmission. Among the maternal factors, high HIV RNA level at delivery, low maternal CD4+ cell count and maternal viral properties are all predictors of MTCT, with HIV RNA level being the most consistent one [60]. Among the obstetrical factors, prolonged rupture of membranes [61], preterm delivery [62] and chorioamnionitis are all risk factors of MTCT [63]. Among the neonatal risk factors, premature birth (<35 weeks of gestation) [64] and low birth weight [61] have been shown to be associated with higher neonatal acquisition of HIV infection.
There is also some evidence to support that cigarette smoking and illicit drug use increases the risk of transmission by disrupting the placenta [65]. Intrauterine transmission is supported by case reports where the virus was detected in fetal material at 12 weeks of gestation [66] and in the amniotic fluid [67].

1.4.1.1 Prevention of MTCT

There are some specific interventions which can reduce the risk of MTCT of HIV. In developed countries, women usually take all of the interventions available and these decrease the rate of transmission of the virus to <2% [58]. However, the transmission rate is between 25% and 45% without any of the preventions, Available interventions are as follows:

- Taking ARV therapy during pregnancy, except, if possible, during the first 3-4 months in pregnancy to avoid exposing the fetus to drugs at this early developmental stage [58].
- Providing a short course of prophylactic ARV therapy to the neonate immediately after birth [58].
- Cesarean birth as the delivery mode – Cesarean delivery can be planned to prevent the baby from being directly exposed to the mother’s genital tract secretions or blood. It is usually done at 38 weeks of gestation.
- Refraining from breast-feeding – Since HIV is found in breast milk, it is recommended that HIV-infected women abstain from breast-feeding if milk substitutes are available. Among HIV-infected women who do not breast-feed, the rate of vertical transmission is about 15% to 25% whereas the rate increases to 25% to 45% for those HIV-infected women who breast-feed [68]. An
investigation from Rwanda reported that 5% of the children born to HIV-infected mothers acquire the infection after 3 months of age, substantiating the contribution of breast-feeding in the overall transmission rate [69]. In resource-poor countries where safe water is not available, formula feeding is limited. Other limitations may also be due to the high cost of formula, the fact that infants who are formula-fed may be more susceptible to infections and the fact that formula feeding may identify a women as HIV-infected in some societies [56].

1.4.2 HAART in pregnancy

In pregnancy, it is recommended that HIV-infected women receive antiretroviral treatment for their own health as well as to prevent the virus from being transmitted to their child. Zidovudine (AZT), an NRTI, was the first antiretroviral drug to be approved by the United States Food and Drug Administration (FDA) in 1987 and was initially taken as monotherapy. However, it showed several side effects. Four years later, dual therapy was introduced when several other NRTIs were approved by FDA during the early 1990’s. With the advent of PI’s in the mid 1990’s, combination therapy was introduced and this was called HAART.

Many ARV drug regimens that include AZT, AZT and 3TC, NVP or a combination of these in HAART have been shown to be successful in reducing vertical transmission to < 2% in resource-rich countries [70]. HIV-infected pregnant women are recommended to start ARV during the second trimester of their pregnancy [56]. Different types of combination therapy are given to the infected mother based on factors that play major roles in the progression of the disease, such as the CD4+ count and plasma HIV RNA levels [58]. According to the 2010 international guidelines on
antiretroviral drugs, HAART is recommended for all HIV+ pregnant women in their second trimester, regardless of their HIV RNA levels [41]. The first-line regimen of these pregnant women is AZT+3TC+NVP, a combination which has shown the greatest clinical effect [71]. In addition to exposure to HAART in utero, it is recommended that infants receive oral AZT during the first 6 weeks of life [72]. Transmission of the virus through breastfeeding has been shown to be reduced in women receiving HAART during the period of breastfeeding [73]. In the Mitra plus study, the transmission rate through breast milk was examined in women on AZT, 3TC and NVP and the transmission rate to infants was 0.9% at 6 months, rising to 1.7% in 12 months and 1.9% at 18 months [74].

1.4.3 Risks of ART exposure

The transfer of ARVs from the mother to the fetus is beneficial in preventing vertical transmission of the virus, but it can expose the fetus to drug toxicity resulting in hematologic and hepatic toxicity risks to the infants [75]. In a 2000 European Collaborative study, the total occurrence of congenital abnormalities was 2-3% in infants who had been exposed to ARV in utero (602/3740 infants with exposure to HAART) [76, 77]. Furthermore similar prevalence of congenital abnormalities was seen for infants who were exposed to ARV in the first trimester compared to infants with later exposure [77].

NRTIs are implicated as chemical mutagens due to their ability to incorporate into elongating DNA, which can result in telomere shortening [78], and mitochondrial DNA alterations due to their affinity for mitochondrial DNA polymerase gamma [79]. Certain
haematological toxicities have been associated with ARV exposure, such as anaemia, higher levels of platelets and lower levels of neutrophils in infants exposed to AZT [80]. Other pregnancy-related risks related to HAART exposure in HIV-infected pregnant women include pre-eclampsia [81], insulin resistance, and hyperglycaemia. Moreover, it has been reported that there is an increased risk of gestational diabetes in women receiving PI-containing HAART [82]. There have also been cases of hyperlactatemia during the first few months of children who had been exposed to AZT alone or in combination [83].

Even though the benefits of administering ART during pregnancy outweigh the risks of the side-effects in HIV/ARV-exposed children, both the possible short-term and long-term consequences for the children should be considered.

### 1.5 Telomeres

Telomeres are non-coding nucleoprotein structures present at the ends of eukaryotic chromosomes [84]. These telomere caps prevent the erroneous repair and nucleolytic degradation of chromosome ends and are composed of repetitive TTAGGG sequences that are bound by a protein complex called shelterin [85]. The reverse-transcriptase enzyme of telomerase (TERT), maintains telomere length by adding telomeric repeats de novo to the 3’-OH end of chromosome ends [84]. These G-rich repeats are about 9-15 kb long in humans and progressively shorten with each cell division [86]. Telomere shortening has been associated with several age-related pathologies [87].
1.5.1 Biology of telomeres

Telomeres shorten with each cell division because the lagging strand of DNA synthesis is unable to replicate the extreme 3’ end of the chromosome (“end-replication” problem) [88]. This progressive attrition of telomeres continues until after a finite number of cell divisions after which division ceases. This barrier is known as the ‘Hayflick limit’ where the telomeres reach a critical length at which they enter a stable non-dividing state [89]. This irreversible inhibition of replication is known as replicative senescence and it is usually triggered by DNA that has not been repaired or uncapped telomeres [90].

Without telomeres, the ends of linear chromosomes would be recognized as DNA breaks and trigger the genome repair mechanism resulting in chromosome fusion, enzymatic degradation, deletion and cell cycle arrest. If these broken DNA are not dealt with properly, continuous cell division would lead to unequal distribution of DNA which in turn would cause massive genomic instability [86]. Telomeres thus act as nucleoprotein caps that prevent the erroneous activation of DNA damage responses of the cell. Telomere maintenance is accomplished by the telomerase complex (see section 1.5.1.2), an enzyme that uses the 3’-OH of the G-tail at the end of the telomeres to add new telomeric repeats [91]. Telomerase is present in germline cells, tumor cells and embryonic and some adult stem cells; it is absent from most human somatic cells except for tissues where cells are undergoing constant proliferation, such as keratinocytes of the skin, cells of gastrointestinal tract and blood [92]. With the absence of telomerase, cell division of somatic cells results in progressive shortening of telomeric sequences by 100-200 bp [86].
1.5.1.1 Structure of telomeres

The tandem repeats of mammalian telomeres consist of a 3’ G-rich overhang which is the substrate for telomerase. This G-strand overhang is able to fold back into the double-stranded telomere region, forming a loop called telomere loop or T-loop [93]. T-loops protect the telomeres by making the 3’ end inaccessible to degradation and DNA repair mechanisms [94]. Binding the telomere repeats is a multiprotein complex called shelterin or telosome. This complex consists of several proteins which functions in the protection of chromosomes and in the regulation of telomere length [85]. These components include the Pot1-TPP1 heterodimer, which bind to the G-rich overhang, as well as TRF1 and TRF2 which, with their interacting proteins Rap1 and Tin2, bind to the double-stranded telomeric repeats [95]. These control the access of telomerase to its substrate, as well as prevents chromosome end-to-end fusions [96].

1.5.1.2 Telomerase – Structure and Function

Most eukaryotes contain a universally conserved enzyme complex called telomerase, which is responsible for maintaining telomeric repeats at the ends of chromosomes [97]. It is a ribonucleoprotein that contains two essential components for its catalytic function Figure 2:

- Telomerase RNA (TER or TERC): This segment of RNA is 451 nucleotides long and serves as the template for the synthesis of telomeric repeats by the reverse transcriptase activity of telomerase. The 3’-OH overhang of the telomeres serves as the primer for reverse transcription [98].
Telomerase reverse transcriptase (TERT): The arrangement of this enzyme is usually set up as an analogy of the of a “right hand”, where the fingers and palm is deployed as the RT motif and the short (~150-200 amino acid) C-terminal as the thumb [99, 100]. The RT motif is flanked by a large (~400 amino acid) N-terminal extension, which have been shown to be crucial for both enzymatic activity and regulation of telomerase [100]. The unique characteristic of TERT is that it can synthesize long DNA segments of >100 nucleotides long despite the short RNA template (~8-31 nucleotides) [101].

Figure 2: Structure of the telomerase ribonucleoprotein complex

Elongation by telomerase occurs via repeated extension and translocation steps. First, the 3’ telomeric end is recognized by the telomerase and the 5’ end interacts functionally to the ribonucleoprotein’s anchor site. Elongation by TERT occurs by sequential addition of six nucleotides to the 3’ end of the telomeric DNA substrate and continues until the end of the 5’ region of the template. The RNA then undergoes a
change in structure, initiating the dissociation of the DNA-RNA hybrid from the active site by accelerating the translocation process by telomerase. This causes the 3’ end of the telomeric DNA to come in contact with the 3’ region of the RNA template, allowing the next round of nucleotide addition. The advantage of this translocation reaction is that TERT can repeatedly use the same template region to produce multiple extensions of telomeric DNA [102-104].

1.5.2 TERT and HIV RT

The domain organization in TERT is conserved in reverse transcriptase enzymes from retroviruses [105] (Figure 3). Several structure-function studies suggested that the catalytic mechanism is conserved between HIV RT, TERT and all other RTs [105]. The mechanistic similarity between HIV RT and TERT makes TERT susceptible to NRTIs as well.

Figure 3: Homology between the catalytic subunit of HIV RT and telomerase reverse transcriptase (TERT) (adapted from [105]).
1.5.2.1 Reverse transcriptase inhibitors and telomere shortening

Approximately 85-90% of all human tumors exhibit telomerase activity [106]. Therefore it is hypothesized that telomerase must remain activated for telomere maintenance so that tumor cells can continue their uncontrolled proliferation. In the past several years, there has been a great increase in research on telomerase in order to develop approaches for inhibiting the enzyme, as a therapeutic strategy for the treatment of cancer. However, inhibitors of telomerase may have detrimental effects on other human somatic cells that express telomerase activity [92].

As previously mentioned, two NRTIs that are widely used in HAART for HIV infection are AZT and 3TC. Therefore they are capable of incorporating themselves into the viral DNA and prevent reverse transcriptase from elongating the cDNA chain [45]. However, certain genotoxicity may arise as a result of the chronic use of AZT. These include micronuclei formation, sister chromatid exchanges and chromosomal aberrations [107]. It has also been well-documented that after phosphorylation, AZT can get incorporated into both nuclear and mitochondrial DNA [108].

Similar to the way that AZT terminates viral DNA elongation, it has also been shown to accumulate in telomeric regions in place of thymidine. This inhibits telomerase from adding the next incoming nucleotide, preventing elongation of telomere DNA and causing irreversible telomere shortening [109]. AZT-induced telomeric shortening has been seen in HeLa cells [110], in human transformed JY616 B-cell line [111], in four breast cancer cell lines, in a T4 cell leukemic line, etc [112].

AZT can also specifically get incorporated into the repeats of telomeres causing oxidative DNA damage and loss of telomeric DNA signals [111]. Moreover, AZT can interfere with the processivity of DNA polymerase, negatively affecting the DNA repair
mechanism. Double-strand breaks and oxidative damage together can then result in genomic instability and cell death, inducing an increase in cellular renewal, ultimately leading to increased telomere attrition [111, 113, 114]. The induction of telomere erosion is therefore a possible consequence of the action of NRTIs.

1.5.3 Telomeres and aging

As mentioned above, most human somatic cells do not express telomerase required for the maintenance of telomere length. Normal human somatic cells have a finite replicative life span which is about 50 ± 10 population doublings (“Hayflick’s limit”) as shown by Hayflick’s in vitro culture of human fibroblast cells [89]. Once cells reach this finite number of population doublings, they enter a state of irreversible growth arrest called replicative senescence, a consequence of telomere shortening.

Early studies of telomere length on human skin fibroblasts showed that telomere length declined linearly with age [115]. This type of correlation was also observed in peripheral blood mononuclear cells (PBMC) [116], and in CD4+ and CD8+ peripheral T cells [117]. However, recent reports showed a more complex example compared to the linear relationship observed initially. Frenck et al. found that in peripheral blood cells of young children, telomeres shorten rapidly at a rate of >1 kb/year. This rate reduces at ages between 4 to 20 years and remains relatively stable in the remaining adult years of life [118].

The regenerative capacity of various tissues decreases with age in humans. There is a decrease in stem cell function and a decline in telomere reserves. The function of stem cells usually limits the homeostasis of our organs and this in turn limits our longevity [119]. It is believed that when cells reach a critically shortened telomere
length, they produce a group of proteins that are different from those produced by non-senescent cells and these new set of proteins may change the homeostasis of the tissue [120]. A drop in organ homeostasis may cause a reduced ability to tolerate age-associated stresses. Oxidative stress is one of them and is a stimulus of stress-induced senescence [121]. Oxidative stress can promote cellular senescence by activating cellular checkpoints that are triggered in response to double-strand DNA breaks. Another way in which oxidative stress induces telomere attrition is through the guanine repeats of telomeres. These triple nucleotides are prone to oxidative modifications which cause the telomeres to become more breakable, leading to wearing away of the telomere ends and ultimately senescence [122].

1.5.3.1 Age-associated diseases due to telomere shortening

Short telomeres have been associated with diseases and conditions whose prevalence increases with age. Some of these diseases are associated with mutations in the germ-line for the genes TERT and TERC, which are the two major components of telomerase. Dyskeratosis Congenita (DKC) is an X-linked disorder caused by a mutation in the gene encoding the protein dyskerin, which is one of the proteins that are associated with the telomerase complex [123]. Loss-of-function mutations in TERT and TERC also causes the premature aging syndrome DKC [124]. The leading cause of death for patients with DKC is bone marrow failure followed by progressive fibrosis in the lung and cancer [125]. The bone marrow failure is characterized by aplastic anemia or reduction in the blood cell lineages that give rise to cells of the immune system, as well as impaired tissue proliferation [126].
Markedly short telomere lengths from age-matched controls have been observed in the circulating cells, cultured fibroblasts, or DNA from leukocytes of patients who exhibit DKC and bone marrow failure. These patients have also been shown to carry mutations in genes encoding proteins of the telomerase complex. Such mutations may lead to telomere shortening by decreasing the stability or activation of the telomerase complex, or directly hampering the complex’s enzymatic activity [127].

Various studies have associated short telomere length with premature myocardial infarction [128], dementia and mild cognitive impairment diagnosis [129]. It is interesting to note that, in a mouse model, short telomere even in the absence of mutations in telomerase cause age-associated degenerative defects that mimic those present in DKC patients. Therefore, short telomeres in these mice damage the production of stem cells and lymphocytes which give rise to the age-associated features of combined immunodeficiency [130].

1.5.4 Factors associated with shorter telomere length

Telomere length is a multifaceted trait and there are a variety of genetic, epigenetic and environmental factors that play a role in determining its length [131].

1.5.4.1 Environmental and lifestyle factors

A significant correlation between chronicity of caregiving stress and telomere length have been demonstrated in healthy women, implying that psychological stress is associated with factors that accelerate aging, such as oxidative stress and telomerase activity in PBMCs [132].
Leukocyte telomere length (LTL) is inversely correlated with body mass index (BMI) [133], which in turn indicates the consumption and expenditure of calories. It was observed that women and men with lesser physical activity had shorter leukocyte telomeres (even after adjusting for possible confounders) than those who were more active. The relationship between physical activity and telomeres indicates that a high BMI, a sedentary lifestyle, low socioeconomic status (SES) and cigarette smoking all share common metabolic pathways that influence the dynamics of leukocyte telomeres [134].

Obesity and cigarette smoking are risk factors for telomere attrition and therefore age-related diseases since they are both associated with oxidative stress, that causes telomere shortening, and inflammation, which increases white blood cell turnover [135, 136]. BMI, leptin concentration (a marker and regulator of body fat) in serum and packs-year of cigarettes smoked all have a negative correlation with telomere length [133]. Socioeconomic status plays an important role on health and is associated with a shortened life expectancy by predisposing to aging-related diseases [137]. Cherkas et al. showed that women with a lower SES had shorter white blood cell telomere lengths than women with higher SES [138].

1.5.4.2 Diet

Oxidative stress and inflammation are involved in shorter telomere steady-state length and diet affects both these processes; however there has been limited studies exploring the importance of dietary intake on telomere length. A recent cross-sectional study has shown that telomere length is inversely associated with the intake of polyunsaturated fatty acid and waist circumference in women [131]. This may partly
help to explain potential pathways by which diet and body composition are involved in the development of certain age-related outcomes such as cardiovascular disease, type 2 diabetes and some cancers [139-141]. The inverse relation between obesity and LTL explains the fact that as adipocytes accumulate, they increase oxidative stress causing the inflammatory cytokines to be deregulated [142]. Since telomeric DNA is not as capable of DNA repair compared to genomic DNA, this would accelerate telomere shortening during cell cycle leading to replicative senescence [143]. A positive correlation has been observed between LTL and the intake of dietary fiber, especially cereal fiber and whole-grain, suggesting that plant-based diets may influence telomere length by exerting antioxidant and anti-inflammatory mechanisms [131].

### 1.5.4.3 Heredity

Several studies have shown that heredity plays an important role in telomere length [144]. Many genes have been reported to influence telomere length [145, 146] and the heritability ranges from 36% to 90% [147]. A significant and positive association was present between the telomere lengths in PBMCs of fathers and their off-springs, suggesting that paternal inheritance is contributing to telomere length [148]. One study suggested that telomere length is linked to the X-chromosome given that an association was found between telomere length in mothers and off-spring and between fathers and daughters but not between fathers and sons [149]. This association may be due to a group of genes that reside on the X chromosome (such as DKC1 and AGTR2) that have been shown to play a role in the function of telomerase.

In contrast to what is seen in other cell types, sperm telomere length increases with age [150] and is transmissible. Unryn et al. showed that paternal age at conception
was positively and significantly associated with both the daughter and son’s blood telomere lengths. This supported the importance of a father-linked inheritance pattern demonstrated in more recent studies. However, this group found no significant correlation between maternal age at conception and the telomere lengths of the male and female offspring. Thus, although there is some inconsistency in the literature, paternal age is evidently linked to telomere length of children and could help to explain the variability of telomere lengths observed at birth [151].

1.5.5 Telomere measurement

It is important to assess the telomere length in order to understand the biological and clinical implications of telomeres on cancer, genetic abnormalities and age-related diseases [152, 153]. There are several methods to measure the length of telomeres. These include terminal restriction fragment (TRF) southern blot, single telomere elongation length analysis (STELA), quantitative polymerase chain reaction (qPCR) and histochemical methods such as quantitative fluorescence *in situ* hybridization (Q-FISH) and flow-FISH.

1.5.5.1 TRF analysis by Southern blot

The quantitative measurement of TRFs can be achieved by southern blot, a technique first described by Southern in 1975. Briefly, genomic DNA is digested by two restriction enzymes which are frequent cutters and separated by agarose gel electrophoresis. Then it is usually transferred to a membrane and hybridized by telomere-specific labeled probes. The size and abundance is then quantified by
densitometry [146]. This method can be used on all cells and tissues of humans as long as an adequate amount of sample is available (~1ng per sample) and the TRFs are within the marked range of the method [154].

Although widely used, this method has several disadvantages. The DNA that is extracted for analysis should be unfragmented and pure, which is sometimes difficult to achieve due to the high viscosity and molecular weight of DNA. Moreover, the TRF does not provide the actual length of the telomere since it also takes into account the unknown length of the subtelomeric region [146]. It is also a labor-intensive and costly method. However, improvements have been made by combinations with other methods and southern blot still remains the most commonly used methods of telomere length measurements, and is the gold standard for telomere assessment [155].

1.5.5.2 FISH

FISH (fluorescence in situ hybridization) is a cytogenetic technique that uses fluorescent oligonucleotide probes in order to detect the presence of a desired sequence on a chromosome. The standard procedure of FISH includes preparation of metaphase chromosomes, DNA denaturation, hybridization with synthetic probes, counterstaining and finally visualization by fluorescence microscopy for quantification of the fluorescent signals [156]. The process of quantifying telomere length with the FISH method is known as quantitative-FISH (Q-FISH). Q-FISH was developed by Lansdorp et.al, where peptide nucleic acid oligonucleotide probes are used instead of oligonucleotide probe, for more intense staining of telomeres and enhanced sensitivity. Its advantage over the TRF southern blot is that it requires very small amounts of metaphase cells (<30 cells) compared to the large amount (>100 cells) required for TRF
Some of its disadvantages are that it requires specialized instrumentation, it is relatively costly, time consuming, careful determination of the linear range has to be established, and only a small subset of telomeres can be examined at any one time relative to the bulk methods (e.g. TRF analysis) [158]. Moreover, the controls used only give a relative measurement.

Flow-FISH

Flow-FISH measures telomeres in individual cells by combining FISH with flow cytometry [159]. The basic steps are the same as the standard FISH method except that peptide nucleic acid probes are used and the analysis is done by flow cytometry. Automated multicolor flow-FISH is a fast and sensitive method to measure the average telomere length in cells of human blood such as granulocytes, lymphocytes and natural killer cells [160]. Some of the advantages of flow-FISH are that telomere lengths can be analyzed in both cycling and non-cycling cells instead of the preparation of metaphase chromosomes and the whole process can be done in the same day; these cannot be achieved using the Q-FISH.

However, several features limit the use of Flow-FISH. Intact separated cells are required for flow-FISH-mediated studies making it inappropriate for the study of solid tissues. Granulocytes have a short life span in vivo, making them unstable in ex vivo conditions and urging the sample to be processed as promptly as possible [154].
**1.5.5.3 STELA**

Single telomere elongation length analysis is a PCR-based technique that developed by Duncan Baird in 2003. This technique is used to measure the telomere length at the level of individual chromosomes [161] and being PCR-based, it has a higher resolution than the previously described techniques of telomere length measurement. In contrast to the TRF analysis, specific telomere ends can be targeted by the use of chromosome-specific primers. The procedure consists of the annealing of the ‘telorette’ to the telomeric G-rich overhang. The telorette is a linker that consists of 7 repeats of TTAGGG followed by 20 nucleotides of a non-complementary tail. The linker ligates to the 5’ end of the C-rich strand in the telomere, after which PCR amplification is carried out using the ‘teltail’. This teltail primer is identical to the 20 nucleotide non-complementary region of the telorette. Along with the teltail, a chromosome-specific primer is also included in the reaction for the upstream subtelomeric region [161].

The disadvantage of this method is that telomeres greater than 25 kb in length will not be amplified since this is a PCR-based technique. This method is also time-consuming and has a low-throughput, making it unsuitable for large population studies.

**1.5.5.4 qPCR**

The methods described above have several constraints, such as the requirement of large amounts of sample and time, and the existence of subtelomeric restriction site polymorphisms which may confound the measurements and cause inter-individual variation in the average length of the telomere. Some of these constraints have been overcome by the development of a simple and high-throughput quantitative real-time
PCR-based method that allows the measurement of relative telomere lengths in a wide range of samples [162]. This fluorescence-based qPCR exhibits the capacity to detect and quantify very small amount of nucleic acids (50 ng per sample) in large cohort samples from different sources [163, 164].

Cawthon published the technique in 2002. His strategy involved the hybridization of a pair of oligonucleotide primers to the repeats of telomeres and generating a product T (for telomere) that is normalized to a single copy gene (S). The oligonucleotide primers that were designed for this study specifically amplify telomeric repeats and avoids the formation of primer-dimer products as explained in Figure 4. The T/S ratio is demonstrated to be proportional to the average telomere length in the sample. The reference DNA sample is used as a standard to which all the experimental samples are compared. The amount of telomere repeats and the amount of single copy gene in each experimental sample is expressed within a level of dilution of the reference DNA so that the experimental and reference DNA are comparable in terms of the number of PCR cycles that are required to produce a specific amount of telomere product during amplification [162].

The qPCR determines the control point for each sample well. The control point is the cycle number at which the fluorescence crosses a threshold level which lies above the baseline fluorescence [165]. The linear plot of the control point against the amount of input DNA allows the quantification of unknown samples by comparing them to a standards curve of serial dilutions of the reference DNA that was amplified in the same plate [162].

The advantages of the qPCR assay are as follows:

- Relatively simple
- High throughput
• Requires minute amounts of samples
• Rapid
• Cheap
• Able to assay partially degraded samples
• Eliminates the formation of primer-dimers, which is a problem with the previously mentioned telomere length assays, such as the Southern blot.

However, like all other methods, qPCR is not without its drawbacks. One major disadvantage is that due to its sensitivity, very small changes in the efficiency of the PCR in either the telomeric repeat or the single copy gene may cause a considerable impact on the ratio. Moreover, the lack of good reference DNA can make the measurement of telomere length difficult. In spite of these drawbacks, qPCR has swiftly spread among telomere epidemiologists due to the technique’s ability to overcome the shortcomings of the previously mentioned telomere length assays [154, 162].
Figure 4: Schematic representation of the annealing of primers tel 1 and tel 2 to genomic DNA (A) and to one another (B) during the first round of PCR.

(A) Primers Tel 1 and Tel 2 annealing to DNA. In both of the hybridizations between primer and template, every sixth base is mismatched and the last five bases at the 3’ ends of the primers are perfectly matched with the bases opposite to them, allowing the addition of bases by DNA polymerase for elongation.

(B) Primers annealing to one another. The primers contain a repeated pattern of six bases composed of four consecutive paired bases followed by two mismatched bases. The base at the 3’ end of each primer cannot hybridize with the base opposite to it, blocking the incorporation of incoming bases by DNA polymerase and preventing primer-dimer formation. (Figure adapted from Cawthon et al. [162].)
Several telomere studies have provided insights into the function of T and B cells in HIV-infected individuals. In a longitudinal study, PBMCs were collected over a nine-year period and it was observed that the telomere lengths of total PBMCs from HIV-infected individuals exhibited accelerated shortening compared to age-matched seronegative controls [166]. These data indicated that HIV infection was associated with increased cell division of cells of the immune system. Therefore, several groups carried out telomere measurements on two T cells subsets to examine the turnover and exhaustion of CD4+ and CD8+ T cells in HIV-infection. Telomere length shortening was consistently observed within the CD8+ T-cell subset whereas minimal or no telomere shortening was seen in the CD4+ subset. Within the CD8+ subset, telomere shortening could be observed in all of the cells that lacked CD28 expression. The telomere length of these CD8^+ CD28^- T cells were the same size as the lymphocytes of centenarians, suggesting that immunological aging is accelerated during HIV infection [167].

The lack of telomere shortening in CD4+ T cells is suggestive of high turnover rates during HIV infection because the massive destruction or apoptosis of activated CD4+ cells is replaced by naïve T cells which would have longer telomeres than memory CD4+ T cells [117]. On the other hand, the CD8+ cell subset telomeres shorten because the CD8+ T cell population expands due to antiviral responses and activation of cytokines. This occurs as a “bystander” effect to compensate for the loss of CD4+ T cells during HIV infection. Therefore these cells would have shorter telomeres because of extensive cell division [167]. Pommier et al. have shown that the telomere lengths of

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2 CD28 is a costimulatory molecule whose loss of expression on cells surfaces occurs when T cells reach cell cycle arrest after several rounds of proliferation.
B cells also shorten, which is compatible with hyperactivation and antibody production that is observed during HIV infection [168].

1.5.7 Telomeres in cord blood and neonates

There have been a few telomere studies on cord blood from full-term neonates [169, 170]. It is suggested that telomere length measurements in umbilical cord blood of preterm (<37 weeks of gestational age) and full-term neonates (>37 weeks of gestational age) would give insight into whether gestational age plays any role in influencing telomere length [171]. The umbilical cord contains many hematopoietic progenitor cells that have longer telomeres than those from adult bone marrow [169]. In one study, the telomere lengths in the leukocytes of cord blood samples from preterm and full-term neonates were compared and no significant differences in the TRF of the two groups were observed. Additionally, a significant and quick decline in telomere length was seen in the preterm neonates who were between 27 and 32 weeks of gestational age, whereas there was no significant loss of telomeres between 33 and 42 weeks of gestation. Friedrich et al. concluded that the rapid attrition of telomeres before 32 weeks of gestation is most likely due to the maximal rate of proliferation of hematopoietic progenitor cells during that period [171].

The mean telomere length of both early and mature hematopoietic cells decreases with age and fetal hematopoietic stem cells (HSCs) have the ability to proliferate more than adults HSCs [172]. A recent longitudinal study compared the mean telomere length of fetuses with increasing age and compared them to gestational age-matched preterm infants, both over a similar period of time. Consistent telomere loss was observed only in the age-matched preterm infants and not in the fetuses,
suggesting that the maintenance of telomere length work via different mechanisms in HSC in fetal life compared to post natal life [173]. This has been supported by the observation that fetal liver HSC contains higher levels of telomerase than the HSCs of adult bone marrow [174].

It is well established that maternal diabetes gives rise to abnormal intrauterine environment that may hamper the long-term health of the offspring [175] and it has been suggested that the outcomes of this may be brought about by intrauterine chromosomal telomere attrition [176]. Cross et al. have demonstrated that telomere shortening occurs in patients with diabetes [177]. Although no significant difference was found in cord blood telomere length between gestational diabetic pregnancies and control pregnancies, there was a significant upregulation in the activity of telomerase in cord blood mononuclear cells of the gestational diabetes group. It was suggested that this upregulation of telomerase in the cord blood could indicate in utero telomere maintenance in response to oxidative stress and telomere DNA damage or potential telomere attrition in utero [178].
2 Blood telomere length in infants and their HIV-infected mothers exposed to antiretroviral therapy during pregnancy

2.1 Introduction

It has been described in section 1 that HIV-infected pregnant women are treated with NRTI-containing HAART regimens. This approach has been extremely successful in reducing the risk of HIV transmission to their infants. However, little is known about the potential adverse effects of exposing the developing fetuses and infants to these drugs. As nucleoside analogues, NRTIs may inhibit telomerase, leading to accelerated telomere attrition [179]. Telomere loss can eventually lead to critically short telomeres, chromosome instability and cellular senescence or apoptosis. Short telomeres have been associated with aging and the development of age-associated diseases. NRTIs have been shown to cross the placenta and accumulate in the amniotic fluid [180], therefore the developing fetuses may be at risk of telomerase inhibition and telomere shortening in utero. Developing embryos and fetuses undergo rapid proliferation and differentiation (therefore intense DNA replication) during embryogenesis and organogenesis. Since telomerase is expressed in rapidly dividing embryonic and fetal tissues, developing fetuses may be especially susceptible to telomerase inhibition by NRTI compared to adults where cell proliferation is less rapid and telomerase less expressed. Therefore it is important to assess the potential long-term impacts of the antiretroviral drugs on exposed uninfected infants.
2.1.1 Hypothesis

We hypothesized that infants born to HIV+ mothers and exposed to HAART or ART *in utero* will have shorter average telomere length than unexposed infants who are born to:

1) HIV+ unexposed mothers (in the SJ cohort)
2) HIV- unexposed mothers (in the Pregnancy cohort)

2.1.2 Funding and ethical approval

Ethical approval for this study was obtained from the Research Ethics Boards of the University of British Columbia and from the Hospital Research Review Committee of the Children’s and Women’s Health Centre of British Columbia (H03-70356 and H04-70540). When appropriate, amendments were filed to include telomere DNA assays. All study participants (mothers) provided informed consent.

The grant that funded this study was the “CIHR Emerging Team Grant in HIV Therapy and Aging” (CIHR-#HET-85515, principal investigator: Hélène Côté).

2.2 Methods and materials

2.2.1 Study design and population

This study was a retrospective/prospective examination of telomeric DNA length from whole blood samples that were collected from pregnant women and their infants.
2.2.2 Study cohorts

Infants and their mothers were enrolled from two Canadian sites, Centre de Recherche du CHU Sainte-Justine (Montreal) and Children’s and Women’s Health Centre of BC (Vancouver). The Centre de Recherche du CHU Sainte-Justine in Montreal is involved in the care and management of mother, child and adolescent health. The Oak Tree Clinic in Vancouver takes care of all identified HIV-infected children in BC by monitoring them during the first years of life. It is also the reference centre for all HIV-infected pregnant women in BC.

The cohort from Sainte-Justine (referred to hereafter as SJ) collected samples from pregnant women and their infants between 1990 and 2000 whereas the cohort from BC, (referred to hereafter as Pregnancy) collected samples spanning 2005-2009.

A summary about the two independent cohorts:

1. SJ is a retrospective cohort which included pregnant women who were HIV- but ART-unexposed, as well as HIV+ women treated with mono-, dual- or triple-therapy during their pregnancies. Dried blood spot samples were collected from the mothers and their infants.

2. The Pregnancy cohort is a prospective cohort (initially named PR and funded by the Canadian Foundation for AIDS Research (CANFAR), principal investigator: Deborah Money) and later continued as CARMA-1 and funded by the Canadian Institute of Health Research (CIHR), nominated principal investigator: Hélène Côté. PR and CARMA-1 included HIV+ HAART-exposed and HIV- pregnancies (unexposed controls). Blood was collected from these pregnant women around 18, 24-28, 32-36 weeks of gestation, at delivery and 6 weeks post-partum. Infant
blood was collected at the same time as the heelprick blood collection for newborn genetic testing. Placental tissue and cord blood were also collected at the time of delivery. The control samples were collected in a similar pattern; however, blood was not collected from the mothers at the time of delivery.

An amendment was filed and approved for using these samples for the telomeric DNA assay. A parent or guardian gave informed consent as well.

**Study Groups**

Samples were used for this study if available from both mother and infant and if they met the following criteria:

For SJ cohort infants:
1) Born to HIV⁺ mothers
2) HIV-uninfected
3) A dried blood spot sample was collected 0-6 weeks after birth

For SJ cohort mothers:
1) HIV⁺ and pregnant
2) A dried blood spot was collected in pregnancy or shortly after delivery

For Pregnancy cohort infants:
1) Born to HIV⁺ mothers treated with HAART in pregnancy
2) HIV-uninfected
3) A whole blood sample was collected within 7 days of birth

For Pregnancy cohort mothers:
1) HIV⁺ and treated with HAART in pregnancy
2) A whole blood sample was collected at last visit but >7 days before delivery
Unexposed Controls

There were no uninfected controls in the SJ cohort. For the Pregnancy cohort, HIV-uninfected mothers and their infants were recruited through poster display at the Children’s and Women’s Health Centre of BC and at the private doctor’s office of one of the co-investigators in City Square Shopping Centre, Vancouver BC. In addition, women with addiction issues giving birth at the Fir inpatient unit at Children’s and Women’s hospital were approached directly by research staff offering enrollment as controls in the study.

Inclusion criteria for Pregnancy cohort control mothers and infants:

1) Mother not HIV-infected and whole blood sample was collected at last visit but >7 days before delivery
2) Infant whole blood was collected between 0-7 days of life

2.2.3 Method of collection

2.2.3.1 Samples used

For the pregnancy cohort, the maternal venous blood samples were collected in ACD BD Vacutainer blood collection tubes and frozen as whole blood, whereas for infants, heel-pricked blood samples were collected in EDTA pediatric blood collection tubes.
For the SJ cohort, some maternal and infant venous blood samples were collected in EDTA and dried blood spots prepared from that, whereas other infant samples were direct heel-prick dried blood spots.

The following two tables show a summary of the three cohort samples and the clinical study population, respectively:

**Table 1: The Cohort samples**

<table>
<thead>
<tr>
<th></th>
<th>SJ cohort</th>
<th>Pregnancy cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PR</td>
</tr>
<tr>
<td><strong>Infant blood</strong></td>
<td>0-6 weeks</td>
<td>0-7 days</td>
</tr>
<tr>
<td><strong>Maternal blood</strong></td>
<td>Near delivery (2.6 ± 4.0 weeks)</td>
<td>Last visit prior to delivery (2.9 ± 1.9 weeks prior)</td>
</tr>
<tr>
<td>Infants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood origin</td>
<td>Heel DBS</td>
<td>Heel WB</td>
</tr>
<tr>
<td>Groups</td>
<td>HIV/ART-exposed, HIV/ART-unexposed</td>
<td>HIV/HAART-exposed, Unexposed Controls</td>
</tr>
</tbody>
</table>
Table 2: Summary of clinical study population

<table>
<thead>
<tr>
<th>HIV Status of Mother</th>
<th>Pregnancy treatment</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SJ</td>
</tr>
<tr>
<td>HIV-</td>
<td>Unexposed Controls</td>
<td>-</td>
</tr>
<tr>
<td>HIV+</td>
<td>ART-unexposed</td>
<td>39</td>
</tr>
<tr>
<td>HIV+</td>
<td>Monotherapy (AZT)</td>
<td>45</td>
</tr>
<tr>
<td>HIV+</td>
<td>Dual therapy (AZT+3TC)</td>
<td>21</td>
</tr>
<tr>
<td>HIV+</td>
<td>Triple therapy/HAART (2 or more NRTI + PI/NNRTI)</td>
<td>15</td>
</tr>
</tbody>
</table>

The following table represents the divisions of the groups for the mothers and their infants in both the cohorts by which they will be referred to hereafter.

Table 3: Group divisions for mothers and their infants according to their therapy status

<table>
<thead>
<tr>
<th></th>
<th>Mothers</th>
<th>Infants</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ</td>
<td>HIV+/ART-exposed</td>
<td>HIV/ART-exposed</td>
</tr>
<tr>
<td></td>
<td>HIV+/ART-unexposed</td>
<td>HIV-exposed/ART-unexposed</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>HIV+/HAART-exposed</td>
<td>HIV/HAART-exposed</td>
</tr>
<tr>
<td></td>
<td>Unexposed controls</td>
<td>Unexposed controls</td>
</tr>
</tbody>
</table>
2.2.3.2 Extraction of DNA

From the whole blood collected, total genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the protocol specifications made by the manufacturer. The only two changes that were made while following the protocol were as follows:

1) The sample added to the microcentrifuge was halved and diluted with PBS to obtain the final volume; that is, instead of adding 200 μl of the blood sample as per the protocol, 100 μl of blood sample was mixed in 100 μl of PBS.

2) Before the final incubation, the volume of buffer AE added to elute the DNA was half of that mentioned in the protocol or 100 μl.

2.2.3.3 Data collection and clinical laboratory testing

Information that was collected for all cohorts included maternal demographics and antiretroviral history, pregnancy history, non-HIV medications, antenatal antiretroviral use and maternal and infant laboratory results. Clinical records aided in the extraction of this data. The data was stored in a database after collection through confidential questionnaire.

As part of a standard routine care, the CD4+ count and the HIV plasma viral load of the HIV+/HAART-exposed mothers were evaluated prior to delivery.
2.2.3.4 Telomeric DNA assay

This is an *in vitro* assay that measures the relative average telomere length (ATL). Genomic DNA extracted from peripheral blood were used to measure the average telomere length on all available samples by a quantitative real-time polymerase chain reaction method. This assay involves the hybridization of a pair of oligonucleotide primers to the telomere repeats to generate a product that is normalized to a single copy gene (Accessory subunit of polymerase gamma, ASPG, in this case). This relative Telomere/Single copy gene ratio in extracted DNA is demonstrated to be proportional to the average telomere length in a cell [162]. Both telomeric DNA and nuclear DNA are quantified from the total DNA extract. This assay can be applied to DNA extracted from a number of tissues including whole blood, dried blood spots, placenta, cord blood, PBMCs, etc.

The dye SYBR Green is used as a nucleic acid stain for quantification. It binds to double-stranded DNA in most cases and forms a complex that absorbs blue light and emits green light. The advantage of this dye is that it is safer to work with and unlike ethidium bromide, does not have the hazardous waste disposal problem.

The two oligonucleotide primers (tel 1 and tel 2) designed for the purpose of telomeric DNA amplification and quantification by qPCR bind specifically to telomeric DNA repeats without forming primer dimers. This is because the primers are designed in such a way that they have two mismatches after every four matched bases and the 3'-end of each of the primers is also mismatched, hampering polymerization with the incoming nucleotide [162] (*section 1.5.5.4, Figure 4*).

The telomeric DNA assay used for this study was adapted from the procedure by Cawthon *et al.* This assay was also validated and calibrated by assaying a wide set of
blood samples that had been characterized by Lansdorp Flow-FISH method previously (Figure 5).

**Cord blood ATL (N = 30)**

![Graph showing correlation between Flow-FISH and qPCR ATL](image)

\[ y = 0.7962x + 6.4087 \]

\[ R^2 = 0.6397 \]

\[ P < 0.0001 \]

**Figure 5:** Scatterplot showing that the qPCR method correlates well with Flow-FISH

QPCR assay measures relative ATL. The absolute ATL can be estimated using the equation in Fig 5. The qPCR assay is adapted from Cawthon *et al.* and the equipment used for quantitative PCR in the Côté laboratory is the Roche Light Cycler 480.

Materials used for the procedure include:

- LC 480 SYBR Green 1 Master kit (2X conc.) supplied by Roche (Catalogue no. 04707516001). As per the protocol, this ready-to-use hot-start PCR mix contains the following components:
  - FastStart Taq DNA Polymerase
o Reaction Buffer
o dNTP mix (with dUTP instead of dTTP)
o SYBR Green 1 dye
o MgCl₂

- The Primers Tel1b, Tel 2b, ASPF3F and ASPG4R were purchased from Integrated DNA Technologies (IDT) and they were all HPLC (High Performance Liquid Chromatography) purified (Table 4).

**Table 4: List of primers used in the telomeric DNA assay**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Tₘ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tel 1b</td>
<td>5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT-3'</td>
<td>68.3</td>
</tr>
<tr>
<td></td>
<td>TGGGTT TGG GTT TGG GTT-3'</td>
<td></td>
</tr>
<tr>
<td>Tel 2b</td>
<td>5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT-3'</td>
<td>68.0</td>
</tr>
<tr>
<td></td>
<td>TACCCCT TAC CCT TAC CCT-3'</td>
<td></td>
</tr>
<tr>
<td>ASPG 3F</td>
<td>5'-GAG CTG TTG ACG GAA AGG AG-3'</td>
<td>55.7</td>
</tr>
<tr>
<td>ASPG 4R</td>
<td>5'-CAG AAG AGA ATC CCG GCT AAG-3'</td>
<td>54.9</td>
</tr>
</tbody>
</table>

The stock oligo for each of the primers was dissolved in 10 mM Tris HCl pH 8.0, and a 100 μM freezer stock was prepared using DNase and RNase-free water.

- 30K Megapool (Megapool = 24 male volunteers donated blood that was extracted and pooled. The pool was diluted to an ASPG copy number of 30,000 and named 30K megapool). Six serial 1:5 dilutions (in Buffer AE from QIAGEN) of this reference DNA were used to generate a standard curve. Buffer AE is used

\(^3\) Tₘ is the temperature at which a double-stranded DNA or RNA molecule denatures into separate single strands.
to elute DNA during its extraction. It has a pH of 9.0 and contains 10mM Tris-Cl and 0.5 mM EDTA. The study DNA samples were quantified by comparison with this standard curve.

A summary of the major components and their amounts used in the LightCycler 480 qPCR for the telomere reaction is shown in Table 5.

Table 5: Summary of the components for the qPCR assay

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Conc.</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC 480 SYBR Green 1 Master</td>
<td>2X</td>
<td>1X</td>
</tr>
<tr>
<td>Primer 1 (Tel 1b)</td>
<td>10 μM</td>
<td>0.3 μM</td>
</tr>
<tr>
<td>Primer 2 (Tel 2b)</td>
<td>10 μM</td>
<td>0.9 μM</td>
</tr>
<tr>
<td>ASPG Primer Mix</td>
<td>10 μM</td>
<td>1 μM</td>
</tr>
</tbody>
</table>

The amount of each component required was calculated to allow each reaction to take place in a final volume of 10 μl. For each well in a 96-well plate, the total volume of the Master Mix loaded was therefore 8 μl and the amount of sample DNA was 2 μl. The standard DNA and its dilutions were loaded in seven wells of the same 96-well plate. One well served as a negative control into which only the Master Mix and Buffer AE were added. The internal controls used were Tel high and Tel low which are DNA from a newborn infant and an adult respectively, and were loaded into two separate wells on each plate run. The rest of the wells contained unknown samples in duplicates. Once the loading was done, the plate was centrifuged at 1500 rpm for 2 minutes and then loaded into the Light Cycler 480. The qPCR conditions for telomeric and single copy nuclear DNA are summarized in the following table.
**Table 6:** Summary showing the telomere and ASPG qPCR conditions

<table>
<thead>
<tr>
<th>Program</th>
<th>No. of cycles</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Telomere</td>
<td>ASPG</td>
</tr>
<tr>
<td>Preincubation</td>
<td>1</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>Amplification</td>
<td>45</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>Melting Curves</td>
<td>1</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>45 to 95</td>
<td></td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

The figure below shows an illustration of the qPCR assay.

![Figure 6: Summary of qPCR assay](image-url)
2.2.4 Statistical analysis

Prior to the initiation of this study, we conservatively assumed an inter-individual coefficient of variation (CV=SD*100/mean) of 10% for the blood cells average telomere length, based on newborn telomere length data published by others who reported a CV of 6% [171]. We assumed that if our qPCR-based assay has an inter-assay CV of 10%, we should have 99% power to detect a 15% shortening in telomere length, with type 1 error (one-sided) of 0.05 and type II error of 0.20 (power 80%) between ARV-exposed infants and ARV unexposed infants.

Analysis of covariance (ANCOVA) was used to investigate the differences in ATL between exposed and unexposed (SJ) or exposed and control (Pregnancy) infants and their mothers. The following covariates for the SJ and Pregnancy cohorts were included to determine which may be independently associated with shorter telomeres.

Covariates

SJ cohort:

- Maternal age
- Gestational age
- Smoking (cigarette or marijuana (MJ)) ever in pregnancy
- Recreational drugs and/or methadone (ever in pregnancy)

In a linear regression model, additional possible predictors included:

- Duration of ART in pregnancy
- Maternal CD4 count (at last pre-delivery visit, when available)
- Maternal plasma HIV viral load (at last pre-delivery visit, when available)
- Age of infant at the time of sample

**Pregnancy cohort:**

The covariates included for the Pregnancy cohort were the same as those for the SJ cohort but with ethnicity and the duration of pre-pregnancy HAART added. The recreational drugs included heroin, cocaine, crack, crystal meth, ecstasy, benzodiazepine and opioids. The additional possible predictors that were included in the linear regression model were the same as those for the SJ cohort; in addition HIV duration at delivery and Hep C ever in pregnancy were included.

Maternal and gestational age were hypothesized to be a covariate because blood cell telomere length shortens with age [115, 181]. Smoking (cigarettes or marijuana), opioids (such as heroin), cocaine and amphetamines (such as crystal meth and ecstasy) were also included as covariates since they have been shown to be associated with oxidative stress [182-184]. Telomere attrition by oxidative stress occurs through the downregulation of TERT expression, which is regulated by redox-sensitive transcription factors [185-187]. Besides some of these molecules of oxidative stress, methadone can readily cross the placenta as well, but rarely results in birth defects [188].
2.3 Results

2.3.1 Study population

Study subjects were from both the prospective Pregnancy (PR and CARMA-1) cohort and the retrospective SJ cohort. The Pregnancy cohort enrolled a total of 144 mother/infant pairs, with PR and CARMA-1 comprising 120 and 24 subject pairs respectively, at the time of study. The SJ cohort enrolled a total of 142 mother/infant pairs.

Among the initial mother/infant pairs enrolled in the PR cohort, 40 pairs were not included in the study for several reasons: lost to follow-up (N=14), infant blood sample processing error (N=8), infant sample collected > 7 days old (N=8), fetal demise (N=2), misplaced infant sample (N=1), no in utero HAART exposure (N=1), mother sample not collected within the time criteria (N=2), whole DNA lost during centrifugation (N=2), subject withdrew from study (N=1). One eligible participant was mistakenly not included (N=1). Among the CARMA-1 subjects, 4 mother/infant pairs were excluded for various reasons as well: loss to follow-up (N=3) and only DBS available (N=1). Infant blood sample processing error referred to subjects whose EDTA blood was processed to plasma instead of being frozen as whole blood. Loss to follow-up referred to subjects who moved, delivered at a different hospital than Children’s and Women’s Health Centre of BC or were missed.

In the end, out of the remaining pairs in the Pregnancy cohort (PR and CARMA-1), 56 HIV/HAART exposed (41 PR and 15 CARMA-1 subjects) and 43 control (38 PR
and 5 CARMA-1 subjects) mother/infant pairs were included in the telomere study (i.e. assayed and analyzed).

From the Pregnancy study cohort, a total of 86 study and control cord blood samples from PR (N=67) and CARMA-1 (N=19) were included out of the 104 subjects who were enrolled and eligible for this study. Among these, no cord blood was collected for 18 of the subjects, usually because the research staff was not able to be there in time for various reasons. Of the initial mother/infant pairs enrolled in the SJ cohort, 12 mother/infant pairs were excluded from the study for the following reasons: unused IDs (N=2), no sample/insufficient sample (N=3) and infant sample > 6 weeks old (N=7).

After the samples were assayed, it was realized that 10 of the mothers had more than one pregnancy represented and only the first pregnancies were kept, for a total of 120 mother/infant pairs. Among these, 81 subjects were HIV+/ART exposed (45 on AZT mono therapy, 21 on AZT/3TC dual therapy and 15 on HAART) and 39 subjects were HIV-exposed but ART-unexposed.

The following flowchart summarizes the study samples.
Subjects Enrolled

Pregnancy cohort
(Mother+infant samples)
144 pairs:
PR (120) + CARMA-1 (24)

SJ cohort
(Mother+infant samples)
142 pairs

Eligible, therefore assayed and analyzed

Pregnancy cohort
(Mother/Infant pairs):
56 HIV/HAART exposed (41 PR + 15 CARMA-1)
43 unexposed controls (38 PR + 5 CARMA-1)

Pregnancy cohort
(Cord blood):
51 HIV/HAART exposed (37 PR + 14 CARMA-1)
35 unexposed controls (30 PR + 5 CARMA-1)

SJ cohort
81 HIV/ART exposed
(45 mono + 21 dual + 15 HAART)
39 HIV-exposed ART-unexposed

Pregnancy cohort
40 pairs excluded:
8 infant blood sample processing error (plasma)
14 losses to follow-up
8 infant sample collected >7 days old
2 fetal demises
1 misplaced infant sample
1 no in utero HAART exposure
2 mother samples not collected at 32-36 weeks
2 whole DNA lost during centrifugation
1 withdrawn from study
1 error

No cord blood for 18 samples

SJ cohort
22 pairs excluded:
2 unused IDs
3 no/insufficient sample
7 infant samples>6 weeks old
10 mothers twice in the cohort

Figure 7: Flowchart summarizing the study cohorts
2.3.1.1 Demographic and clinical characteristics

Demographics, clinical characteristics and laboratory values for the SJ and Pregnancy cohort are shown in Table 7 and Table 8, respectively.

SJ

For the SJ cohort, all the subjects were HIV+ pregnant women and their infants. One group was exposed to ART in pregnancy (HIV+/ART-exposed group) whereas the other was not (HIV+/ART-unexposed group). None of the infants acquired HIV. Both the HIV/ART-exposed and HIV-exposed ART-unexposed groups for the infants were similar with respect to gestational length, birth weight, infant sex and infant Apgar score at 5 minutes. There was no significant difference between the age of the HIV+/ART-exposed mothers and the HIV+/ART-unexposed mothers, as well as in the ethnicity of the two groups. The majority of the subjects in both the groups were Black-African (59% in the HIV+/ART-exposed group and 62% in the HIV+/ART-unexposed group), the second most common ethnicity being Caucasian (37% in HIV+/ART-exposed and 36% in the HIV+/ART-unexposed groups). Smoking and illicit drug use in pregnancy was similar between both the two groups. None of the mothers were on methadone.

Pregnancy (PR + CARMA-1)

For the pregnancy cohort, one group contained HIV+ pregnant women who were exposed to HAART during pregnancy (HIV+/HAART-exposed) and the other group consisted of HIV- pregnant women (unexposed control). Both groups were similar with
respect to gestational length, infant birth weight, infant sex, infant apgar score at 5 minutes and delivery method. The majority of women delivered vaginally as opposed to delivery by Caesarean section (58% in the HIV+/HAART-exposed group and 70% in the unexposed controls group).

The maternal age of the two groups was similar but a significant difference was found between the ethnicity of the two groups. The HIV+/HAART exposed mothers were more likely to be Caucasian or Aboriginal whereas the unexposed control mothers were more often Caucasian and secondly Asian. Of note, there were no Black-Africans or Hispanics women in the unexposed control group whereas a few subjects from these two ethnicities were present in the HIV+/HAART-exposed group. Active HCV infection based on RNA PCR testing was more common in the HIV+/HAART-exposed mothers than in the unexposed control mothers. However, this data was missing for most of the mothers in the unexposed control group. Smoking, alcohol consumption as well as recreational drug and/or methadone use ever in pregnancy were well balanced between the two groups. Cigarettes and marijuana were considered together under smoking; all the mothers who reported smoking marijuana during their pregnancy also smoked cigarettes. Of all the mothers who received methadone during their pregnancy, 81% (N = 21/26 in both groups) also used illicit drugs.

**HIV+/HAART-exposed group**

For the pregnancy cohort, none of the infants acquired HIV. Out of the 59 HIV+/HAART-exposed pregnant women in this cohort, 11 (19%) conceived on HAART, 9 (15%) started HAART in the first trimester, 33 (56%) in the second trimester and 6 (10%) in the third trimester. In the SJ cohort, 13 (16%) of the HIV+/ART-exposed
pregnant women conceived on ART, 2 (2%) started ART in the first trimester, 55 (68%) in the second trimester and 11 (14%) in the third trimester. After initiation of HAART or ART, all the subjects remained on it throughout the pregnancy. For the Pregnancy cohort, the median [interquartile range] of in utero HAART exposure was 19.3 [14.8 - 29.1] weeks and for the SJ cohort it was 21.4 [15.4 – 37.3] weeks. The majority of the women in the Pregnancy cohort were on AZT+3TC+NFV or LPV (N = 51, 86%) and the women who conceived on HAART were more likely to be on other regimen which did not include AZT (N = 6, 55%). Out of the 81 HIV+/ART-exposed pregnant women in the SJ cohort, 45 (56%) were on monotherapy (AZT), 21 (26%) were on dual therapy (AZT+3TC) and the remaining 15 (19%) were on HAART. In the SJ cohort, 14 out of the 15 women who were on HAART were on the AZT+3TC+NVP regimen.

Thirty women in the Pregnancy cohort received HAART prior to pregnancy and the median [interquartile range] duration was 58.9 [23.3 – 101.5] weeks. For the SJ cohort, only 13 of the women were on ART prior to pregnancy. The median CD4+ count for the women in the Pregnancy cohort was 450 cells/µl, whereas for the women in the SJ cohort the count was 430 cells/µl. Only 10 (17%) of the women in the Pregnancy cohort had a detectable HIV pVL near delivery with a median [IQR] (range) of 321 [80.8 – 440.3] (46 - 1280) RNA copies/ml. Clinical diagnosis showed that the median duration of HIV infection at the time of delivery for the Pregnancy cohort was 3.9 years.
Table 7: Demographic and clinical characteristics of HIV+/ART exposed and unexposed mothers and their infants for the SJ cohort

<table>
<thead>
<tr>
<th>Characteristic or Value</th>
<th>HIV+/ART-exposed (N=81)</th>
<th>HIV+/ART unexposed (N=39)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infant Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational length, weeks</td>
<td>39.0 [38.0 – 40.0] (34.9 – 41.1)</td>
<td>39.0 [37.0 – 40.0] (32.0 – 41.0)</td>
<td>0.17</td>
</tr>
<tr>
<td>Birth weight, kg</td>
<td>3.2 [2.9 – 3.5] (1.9 – 4.2)</td>
<td>3.1 [2.7 – 3.4] (1.9 – 4.3)</td>
<td>0.11</td>
</tr>
<tr>
<td>Male sex</td>
<td>42 (52)</td>
<td>25 (64)</td>
<td>0.21</td>
</tr>
<tr>
<td>Apgar score at 5 minutes</td>
<td>9.0 [9.0-9.0] (7.0 – 10.0)</td>
<td>9.0 [9.0-9.0] (6.0 – 10.0)</td>
<td>0.91</td>
</tr>
<tr>
<td><strong>Maternal Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yrs</td>
<td>28.6 [25.2 – 31.6] (17.3 – 43.9)</td>
<td>26.1 [24.3 – 31.0] (17.8 – 37.4)</td>
<td>0.12</td>
</tr>
<tr>
<td>Maternal Ethnicity</td>
<td></td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>Caucasian</td>
<td>30 (37)</td>
<td>14 (36)</td>
<td></td>
</tr>
<tr>
<td>Black-African</td>
<td>48 (59)</td>
<td>24 (62)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (1)</td>
<td>1 (3)</td>
<td></td>
</tr>
<tr>
<td>Asian + Other</td>
<td>2 (2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal use or consumption ever during pregnancy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>19 (23)</td>
<td>12 (33) (N = 36)</td>
<td>0.26</td>
</tr>
<tr>
<td>Illicit drugs&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>10 (12)</td>
<td>3 (9) (N = 35)</td>
<td>0.55</td>
</tr>
<tr>
<td><strong>ART Exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of maternal ART in pregnancy, weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>17.7 [14.0 – 23.1] (1.1 – 41.1)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>AZT/3TC:</td>
<td>22.9 [14.7 – 24.9] (5.1 – 39.7)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>HAART:</td>
<td>21.4 [15.4 – 37.3] (3.1 – 39.7)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><strong>HIV Clinical Data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detectable HIV pVL, copies/ml, closest to delivery</td>
<td>5546 [1224 – 5739] (205 – 33863)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>CD4+ count&lt;sup&gt;b&lt;/sup&gt;, cell/μl closest to delivery</td>
<td>430 [290 – 575] (33 – 1794)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Data are number N (%) of subjects or median [interquartile range] (range) and N is the total subjects for which data is available (if not available for the entire group).

<sup>a</sup>Smoking includes cigarettes and MJ (Marijuana) and refers to smoking ever in this pregnancy

<sup>b</sup>Normal range is 600-1500 cells/μl

<sup>c</sup>Chi-squared test used for categorical data

<sup>d</sup>Illicit drugs included but were not limited to: heroin, cocaine, crack, crystal meth, ecstasy, benzodiazepine, opioids
Table 8: Demographic and clinical characteristics of HIV+/HAART exposed and HIV- control mothers and their infants for the Pregnancy (PR + CARMA-1) cohort

<table>
<thead>
<tr>
<th>Characteristic or Value</th>
<th>HIV+/HAART- exposed (N=59)</th>
<th>Unexposed Controls (N=44)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infant Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational length, weeks</td>
<td>38.7 [37.7 – 39.9] (31.3 – 41.3)</td>
<td>39.0 [38.1 – 39.9] (28.9 – 41.9)</td>
<td>0.63</td>
</tr>
<tr>
<td>Birth weight, kg</td>
<td>3.1 [2.8 – 3.4] (1.6 – 4.1)</td>
<td>3.1 [2.7 – 3.5] (1.4 – 5.2)</td>
<td>0.39</td>
</tr>
<tr>
<td>Male sex</td>
<td>33 (60)</td>
<td>25 (57)</td>
<td>0.91</td>
</tr>
<tr>
<td>Apgar score at 5 minutes</td>
<td>9.0 [9.0 – 9.0] (7.0 – 10.0)</td>
<td>9.0 [9.0 – 9.0] (5.0 - 9.0)</td>
<td>0.55</td>
</tr>
<tr>
<td>Delivery Method, vaginal birth</td>
<td>34 (58)</td>
<td>31 (70)</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Maternal Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yrs</td>
<td>30.6 [25.6 – 34.7] (18.0 – 42.4)</td>
<td>31.5 [27.2 – 35.5] (22.2 – 43.0)</td>
<td>0.40</td>
</tr>
<tr>
<td>Active HCV infection</td>
<td>11 (30) (N = 37)</td>
<td>2 (11) (N = 18)</td>
<td>0.13</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aboriginal</td>
<td>17 (29)</td>
<td>6 (14)</td>
<td>0.01</td>
</tr>
<tr>
<td>Caucasian</td>
<td>23 (39)</td>
<td>28 (64)</td>
<td></td>
</tr>
<tr>
<td>Black-African</td>
<td>9 (15)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Asian + Other</td>
<td>9 (15)</td>
<td>10 (23)</td>
<td></td>
</tr>
<tr>
<td>Maternal use or consumption ever during pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>36 (63) (N = 57)</td>
<td>22 (50)</td>
<td>0.18</td>
</tr>
<tr>
<td>Alcohol</td>
<td>19 (34) (N = 56)</td>
<td>16 (36)</td>
<td>0.80</td>
</tr>
<tr>
<td>Rec drugs and/or methadone</td>
<td>22 (40) (N =55)</td>
<td>18 (43) (N = 42)</td>
<td>0.78</td>
</tr>
<tr>
<td><strong>HAART Exposure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of maternal HAART in pregnancy, weeks</td>
<td>19.3 [14.8 – 29.1] (1.9 – 41.1)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Duration of pre-pregnancy HAART, weeks</td>
<td>58.9 [23.3 – 101.5] (0.1 – 603.9)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><strong>HAART Combination</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2NRTI + PI (often boosted by ritonavir)</td>
<td>46 (78)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>2NRTI + NNRTI</td>
<td>3 (5)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10 (17)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><strong>HIV Clinical Data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of HIV infection at delivery, yrs</td>
<td>3.9 [1.3 – 6.4] (0.1 – 15.3)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Detectable HIV pVL, copies/ml, at last visit prior to delivery</td>
<td>321.0 [80.8 – 440.3] (46 – 1280)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Undetectable HIV pVL, at last visit prior to delivery</td>
<td>49 (83)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>CD4+ count, cells/μl, at last visit prior to delivery</td>
<td>450 [315 – 665] (90 - 1200)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Data are number N (%) of subjects or median [interquartile range] (range), N is the total subjects for which data is available (if not available for the entire group). Significant p value (<0.05) is highlighted in red.

- Chi-squared test used for categorical data
- Delivery Method- vaginal or Caesarean-section
- Data based on HCV RNA PCR testing
- Smoking includes cigarettes and Marijuana (MJ)
- Recreational drugs included but were not limited to: heroin, cocaine, crack, crystal meth, ecstasy, benzodiazepine, opioids
- Other subjects had no prior exposure to HAART
- Data based on HIV clinical diagnosis date
- Others had no detectable HIV pVL at last visit before delivery
- Undetectable HIV viral load signifies <50 copies/ml
- Normal range is 600-1500 cells/μl
- 1-4 weeks prior but >1day from delivery for PR, last visit before delivery (not always 1-4 weeks) but >7days for Carma
2.3.2 Relative average telomere length comparisons

Whole blood and dried blood spot relative average telomere length was measured by quantitative PCR. For both the cohorts, the average telomere lengths were compared between HIV+/HAART exposed and unexposed mothers and their infants.

2.3.2.1 Covariates

For both cohorts (Pregnancy and SJ), the covariates considered included maternal age at delivery, gestational age, smoking (cigarettes or marijuana) ever in pregnancy and recreational drugs/methadone ever in pregnancy. Ethnicity and Hep C infection were additionally added for the Pregnancy cohort.

Covariate correlations in the SJ cohort

In the analysis for the SJ cohort, some of the covariates mentioned were significantly related to shorter relative average telomere length for all the samples (both the groups) combined. For example, older mothers at birth had babies with longer telomeres (N = 120, r = 0.232, p = 0.01). Smoking was also significantly related to the outcome and mothers who smoked during their pregnancy tended to have babies with shorter telomere lengths (N = 117, r = -0.197, p = 0.03). The telomere length of these mothers tended to be inversely correlated with smoking (N = 117, r = -0.176, p = 0.057). Lastly, among the HIV+/ART-exposed subjects, illicit drug use ever in pregnancy was
associated with shorter maternal average telomere length (N = 116, r = -0.196, p = 0.04).

In linear regression analyses for the HIV/ART-exposed infants, possible predictors of shorter ATL considered included: duration of infant in utero ART exposure and age of the infant at the time of sample. Since telomerase is present in germ line cells, exposure to ART may shorten ova telomeres. This would be passed on to the infants, who would thus be born with shorter telomeres. Therefore we hypothesized that a longer duration of ART may be associated with shorter telomeres in the infant. The list of predictors also included detectable maternal HIV pVL and CD4+ count near (but not always prior) delivery (when available). If the CD4+ count is low or if there is a detectable HIV pVL, it can indicate that the adherence to ART for the particular subject is low or that the virus has developed resistance to the ART regimen taken.

Illicit drug use ever in pregnancy was associated with shorter maternal ATL (N = 116, r = -0.20, p = 0.04) and smoking ever in pregnancy was associated with shorter infant ATL (N = 117, r = -0.20, p = 0.03).

2.3.2.2 Comparing ATLs of mothers and their infants

ANOVA was conducted to compare the average telomere length between mothers and their infants. As expected, infant ATL was significantly greater than maternal telomere length for the SJ cohort (F [1,119] = 104.6, p < 0.001). ANCOVA was subsequently used to control for maternal age, gestational age, smoking ever in pregnancy and recreational drug and/or methadone use ever in pregnancy. After controlling for these covariates, the difference in ATL between mothers and their infants
was no longer significant ($F [1, 89] = 0.02, p > 0.05$) (Figure 8a). A similar result was observed for the Pregnancy cohort (Figure 8b).

**Figure 8a:** Scatterplot showing the comparison between mother and infant ATL for the SJ cohort.

The P value shown is before adjusting for covariates. A significant difference was found between the ATL of mothers and their infants. The difference was no longer significant after adjusting for covariates.
Figure 8b: Scatterplot showing the comparison between mother and infant ATL for the Pregnancy (PR + CARMA-1) cohort.

The P value shown is before adjusting for covariates. A significant difference was found between the ATL of mothers and their infants. The difference was no longer significant after adjusting for covariates.

2.3.2.3 Peripheral blood ATL comparisons between HIV/HAART exposed and unexposed control groups

A one-way between-groups ANOVA was conducted to compare the relationship between either HIV/HAART exposure or no HIV/HAART exposure on ATL in infants and their mothers. As with the previous analysis, ANCOVA was subsequently used to
control for maternal age, gestational age, smoking (cigarette or marijuana) ever in pregnancy and recreational drug use and/or methadone use ever in pregnancy.

For the SJ cohort, no significant difference was found between HIV/ART-exposed or HIV-exposed ART-unexposed infants before (mean ± SD., 7.77 ± 1.54 (N = 81) vs. 7.90 ± 1.76 (N = 39), respectively, F [1,118] = 0.2, p = 0.68) or after adjusting for maternal age, gestational age and smoking (F [1,88] = 0.3, p = 0.57). Similarly, no significant difference was found between the relative ATL of HIV+/ART-exposed or HIV+/ART-unexposed mothers before (5.93 ± 1.36 (N = 81) vs. 6.27 ± 1.21 (N = 39), respectively, F [1,118] = 1.8, p = 0.19) or after adjusting for covariates (F [1, 88] = 2.2, p = 0.15) (Figure 9).
Figure 9: Scatterplot showing the comparison between HIV/ART-exposed and HIV-exposed ART-unexposed infants and mothers for the SJ cohort.

The P values before and after adjusting for covariates are shown. No significant difference was found between HIV/ART-exposed and HIV-exposed ART-unexposed infants or mothers before or after adjusting for covariates.

For the Pregnancy cohort (PR + CARMA-1), the difference between HIV/HAART-exposed or HIV/HAART-unexposed control infants before (mean ± SD, 5.11 ± 1.08 (N = 56) vs. 5.46 ± 1.32 (N = 43,) respectively (p = 0.16)) or after adjusting for covariates
was also not significant (p = 0.67). Similarly, for the mothers, no significant difference was found between HIV+/HAART- exposed or unexposed control mothers before (mean ± SD, 3.43 ± 0.81 (N = 56) vs. 3.49 ± 0.78 (N = 43), respectively (p = 0.70)), or after adjusting for covariates (p = 0.30) (Figure 10).

**Figure 10:** Scatterplot showing the comparison between HIV/HAART-exposed and unexposed control infants and mothers for the Pregnancy (PR + CARMA-1) cohort. The P values before and after adjusting for covariates are shown. No significant difference was found between HIV/HAART-exposed and unexposed control infants or mothers before or after adjusting for covariates.
2.3.2.4 Variation in peripheral blood ATL between different types of sample collection

As mentioned earlier, samples were collected differently for the two cohorts in the study. For the pregnancy cohort, maternal venous blood samples and infant heel-pricked blood samples were collected and frozen as whole blood. On the other hand, for the SJ cohort, dried blood spots were prepared from the venous blood samples and heel-pricked blood samples collected from the mothers and their infants, respectively. All samples were stored at -80°C until used.

Figure 11 a and b show that ATL of mothers and their infants may be influenced by the type of blood sample collection. Relative ATL is generally higher in the SJ cohort compared to the Pregnancy cohort. This figure also shows that there is no significant difference in ATL between mono-, dual- or triple therapy in either mothers or their infants in the SJ cohort.
A. All Maternal Samples:

SJ

Pregnancy

Relative ATL

HIV(+) ART-
unexposed
(N = 39)

HIV(+) AZT
(N = 45)

HIV(+) AZT+3TC
(N = 21)

HIV(+) HAART
(N = 15)

Unexposed
Controls
(N = 43)

HIV+/HAART-
exposed
(N = 56)

Mean
Median
B) All Infant Samples

![Graph showing ATL variation with different types of blood sample collection](image)

**Figure 11**: Maternal (A) and Infant (B) ATL varies with different types of blood sample collection- Dried blood spots in the SJ cohort versus whole blood in the Pregnancy cohort.

### 2.3.2.5 Cord blood ATL comparison between HIV/HAART-exposed and unexposed groups

A one-way between-groups ANOVA was conducted to compare the relationship between cord blood ATL and exposure to HIV/HAART in the Pregnancy cohort. ANCOVA was subsequently used to control for the same covariates. Cord blood ATL was significantly shorter in HIV/HAART-exposed than in unexposed tissue (mean ± SD., 5.65 ± 1.11 (N = 51) vs. 6.24 ± 1.54 (N = 35), F [1, 84] = 4.26, p = 0.04). However, this
difference becomes a trend (F [1, 72] = 3.66, p = 0.06) after controlling for maternal age, gestational age, ethnicity, smoking (cigarette or MJ ever in pregnancy), recreational drug or methadone (ever in pregnancy) and Hepatitis C infection ever (Figure 12).

Figure 12: Scatterplot showing the comparison between HIV/HAART-exposed and unexposed control groups in cord blood for the Pregnancy (PR + CARMA-1) cohort. The P values before and after adjusting for covariates are shown. A significant difference was found between the two groups before adjusting for covariates. However this difference became non-significant after adjusting for the covariates.

2.3.2.6 Controlling for covariates in the HIV/HAART-exposed group

In addition to the previously described covariates, the following predictors were considered in a hierarchical linear regression model: duration of mother’s HAART
exposure prior to pregnancy, duration of infant in utero HAART exposure, a detectable HIV plasma viral load near to delivery. None predicted short telomere length in HIV/HAART-exposed infants (all p values > 0.05). For the mothers, in addition to these predictors, lifetime HIV duration at delivery and CD4+ count near delivery were taken into account. Higher CD4+ count (p = 0.05) and longer HIV duration at delivery (p = 0.02) predicted shorter maternal blood telomere length (N = 52).

In a similar linear regression model for the cord blood, higher CD4+ count (p = 0.02), higher viral load (p = 0.01) and greater maternal age (p = 0.04) predicted longer cord blood telomere length (N = 47).

**2.3.3 Correlations**

Since telomere length is a marker for ageing and a measure of nuclear DNA damage [189], the relationships between maternal age and ATL were explored. Similarly, the possible correlation between maternal and infant ATL was investigated.

**2.3.3.1 Correlation between maternal age and ATL**

No significant correlation was found between the age of the mothers and their ATL for both the SJ cohort (N = 120, r = 0.01, p = 0.95) and the Pregnancy cohort (N = 99, r = 0.02, p = 0.87) (Figure 13). When the association was explored separately for each group in the SJ cohort, there remained no significant correlation between maternal age and maternal ATL in the HIV+/ART-exposed (N = 81, r = 0.001, p = 0.99) or HIV+/ART-unexposed group (N = 39, r = 0.08, p = 0.62). Similarly, the association between maternal age and maternal ATL, whether in the HIV+/HAART-exposed (N = 56,
r = 0.03, p = 0.82) or unexposed group (N= 43, r = -0.02, p = 0.92) was not significant for the Pregnancy cohort.

![Figure 13: Scatterplot showing the lack of significant correlation between the age of all the mothers and their telomere lengths for the SJ and Pregnancy cohorts.](image)

When the association between maternal age and infant ATL was explored, a significant positive correlation for all the samples combined (N = 120, r = 0.23, p = 0.01) was seen in the SJ cohort, but not in the Pregnancy cohort (N = 99, r = 0.03, p = 0.74) (Figure 14). When broken down by groups in the SJ cohort, the correlation between infant ATL and their mothers’ age remained significant in the HIV-exposed ART-unexposed group (N = 39, r = 0.36, p = 0.03) but not in the HIV/ART-exposed group (N = 81, r = 0.19, p = 0.10). Overall though, these correlations remain very weak. For the pregnancy cohort, the correlation between infant ATL and maternal age was not
significant for either the HIV/HAART-exposed (N = 56, r = 0.02, p = 0.88) or the unexposed control (N = 43, r = 0.02, p = 0.92) group.

Figure 14: Scatterplot showing the association between the age of all the mothers and their infants’ ATL for the SJ and Pregnancy cohorts.

A significant correlation was found in the SJ cohort but not in the Pregnancy cohort.

2.3.3.2 Correlation between mother/infant pairs

For the SJ cohort, the correlation between maternal and infant ATL was not statistically significant in either the HIV/ART-exposed or HIV-exposed ART-unexposed group (N = 81, r = 0.18, p = 0.11 and N = 39, r = 0.15, p = 0.35, respectively) (Figure 15).
Figure 15: Scatterplots showing no significant correlation between the ATL of infants and their mothers in either HIV/ART-exposed or HIV-exposed ART-unexposed groups for the SJ cohort.

However, for the Pregnancy cohort, maternal and infant ATL were significantly and positively correlated in both the HIV/HAART-exposed or unexposed controls groups (N = 56, r = 0.41, p = 0.002 and N = 43, r = 0.47, p = 0.002, respectively) (Figure 16).
Figure 16: Scatterplots showing a significant and positive correlation between the ATL of infants and their mothers in both the HIV/HAART-exposed or unexposed control groups for the Pregnancy cohort.

2.3.3.3 Correlations of cord blood ATL with infant and maternal ATL

Cord blood and infant ATL were not statistically correlated in both the HIV/HAART-exposed and HIV/HAART-unexposed control infants (N = 51, r = 0.12, p = 0.41 and N = 35, r = 0.08, p = 0.65, respectively). This was also true when both groups were combined (Figure 17).
Figure 17: Scatterplot showing the relationship between all cord blood ATL and all infants’ ATL for the Pregnancy cohort.

Similarly, there was no correlation between cord blood ATL and maternal ATL in both the HIV+/HAART-exposed and unexposed control groups (N = 51, r = 0.05, p = 0.73 and N = 35, r = -0.05, p = 0.77, respectively) (Figure 18).
Figure 18: Scatterplot showing no correlation between all cord blood ATL and all mothers’ ATL for the Pregnancy cohort.
The following tables summarize the different correlations observed in both the SJ and the Pregnancy cohorts.

**Table 9:** Correlations observed in the SJ and Pregnancy cohorts

<table>
<thead>
<tr>
<th>SJ cohort</th>
<th>r (p), N =120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal ATL</td>
<td>Infant ATL</td>
</tr>
<tr>
<td>Maternal ATL</td>
<td>-</td>
</tr>
<tr>
<td>Maternal Age at delivery</td>
<td>0.01 (0.95)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pregnancy cohort</th>
<th>r (p), N = 99</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal ATL</td>
<td>Infant ATL</td>
</tr>
<tr>
<td>Maternal ATL</td>
<td>-</td>
</tr>
<tr>
<td>Infant ATL</td>
<td>0.44 (&lt;0.0001)</td>
</tr>
<tr>
<td>Maternal Age at delivery</td>
<td>0.02 (0.87)</td>
</tr>
</tbody>
</table>
3 Discussion

Treatment of HIV-infected pregnant women has been successful in reducing the rate of perinatal infection from 25% to <1% [190]. In developed countries, essentially every HIV-infected pregnant woman receives complex HIV regimens during her pregnancy. NRTI’s, the cornerstone of every HAART regimen, can cross the placenta and accumulate in the amniotic fluid, exposing the developing fetuses to drugs. The toxic effects of these drugs are expected to be the greatest during rapid tissue differentiation and proliferation, as is the case for developing embryos and fetuses. This is illustrated by the fact that treatment guidelines in pregnancy recommend to initiate antiretroviral therapy in the second trimester of gestation, to avoid exposure during early embryogenesis. However, pregnancies are often unplanned and women may need HIV therapy for their own health. It is therefore not rare for exposure in the first trimester to occur. For example, in our cohort, approximately 20% of women conceived while on HAART and continued therapy throughout their pregnancy. Very little is known about the potential adverse effects of these drugs on the fetuses and infants. The international community is rapidly increasing the use of HAART to treat HIV-infected pregnancies. Therefore, it is of great importance to investigate the potential toxicity of antiretrovirals on exposed fetuses and infants.

3.1 Telomere findings

We observed that infants have significantly longer average telomere length than their mothers; the difference becomes non-significant after controlling for covariates.
This was an expected finding since telomere length is inversely correlated with age [159]. Normal somatic cells undergo a finite number of cell divisions after which they enter a state of irreversible growth arrest called replicative senescence, which is also involved in the aging process [115, 181]. The length of telomeres in the germ line cells is the starting point of telomeres that are passed on to their offsprings. Beginning with this start point, different factors are involved in changing the telomere length during somatic development. As we age, telomere length shortens with each successive cell division, and this rate of loss has been measured as 50-100 bp per cell division as seen in cultured human fibroblasts or lymphocytes [150]. Therefore, age may most likely be the covariate responsible for taking away the significant difference between mother and infant telomeres. In the SJ and pregnancy cohorts of our study, we observed that mother ATL was 79% and 64% that of their infants, respectively. This maternal-infant ATL difference between the two cohorts may be partly explained by the discrepancy of ethnicity within the two cohorts. In the literature, there is evidence for the role of ethnicity in telomere length [191]. Moreover, variations in telomere length in adults largely depend on genetic and environmental determinants that would begin to exert their effects in utero [192]. Differences in ethnicity may be a major reason of the different genetic and environmental factors, explaining the slightly varying factor by which the mother-infant ATL differs between the two cohorts.

Prior to the beginning of this study, we had conservatively assumed an inter-individual coefficient of variation (CV) of 10% based on newborn telomere length data published by Friedrich et al. [171]. However, we observed a much greater variability of ATL in newborns where ATL variability was similar to that seen in adults. For example, in the SJ cohort, a CV of 19% in mothers and 22% in infants was observed. In the Pregnancy cohort, the CV was 22% in mothers and 24% in infants. Our newborn ATL
observations are consistent with earlier studies which came to our attention after we performed our study, and which reported a variability of telomere length in newborns as large as that observed in adults [192]. These differences in the variability of ATL may be explained by the fact that variation in telomere length between different individuals may be a result of factors such as the telomere length variation at birth, the rate of telomere attrition, or both [192].

The principal finding of this study was that infants who were exposed to ART or HAART in utero did not exhibit shorter peripheral blood ATL than unexposed infants who were either born to HIV+ unexposed mothers (SJ cohort) or HIV− unexposed mothers (Pregnancy controls). Similarly, there was no statistically significant difference in telomere length between HIV+/HAART-exposed and unexposed mothers. The difference remained insignificant after controlling for covariates for both the mother and infant model in the two cohorts. This would suggest that, if HIV/HAART exposure is a risk for telomere attrition, it is less important than some of the variables that were taken into the regression model as predictors of telomere length.

For the SJ cohort, illicit drug use ever in pregnancy was associated with shorter maternal ATL. This may be explained by relating to studies which have shown that neurotoxic effects of the drugs of addiction are associated with oxidative stress and mitochondrial dysfunction, besides several other mechanisms. For example, studies have reported that exposure to cocaine increases the levels of hydrogen peroxide in the brain of rats [193]. Other groups have observed that cocaine usage leads to a decrease in catalase (an enzyme responsible for breaking down reactive oxygen species) activity [194], a decrease in antioxidants and oxidative injury in the brain [195]. Qiusheng et al. have shown that heroin exposure can also lead to a reduction in some enzymes that break down reactive oxygen species, besides causing oxidative damage.
to DNA, lipids and proteins [196]. It is interesting to note that increased neurotoxicity through oxidative stress has been observed in cells that were exposed to both cocaine and HIV gp120 at the same time [197]. As mentioned in section 1.5.3, oxidative stress can lead to telomere attrition since the G-rich region of the telomeric repeats are susceptible to oxidative modifications, causing the telomeres to be prone to breaks [122].

Additionally, in the SJ cohort, smoking (ever) in pregnancy was associated with shorter infant ATL. A recent study by Mozaffarieh et al. showed that those who smoked had a significantly higher rate of single-strand (ss) DNA breaks in leukocytes compared to non-smokers [198]. These ss-DNA breaks resulted from the formation of reactive oxygen species (oxidative stress) and were increased with exposure to cigarette smoke. In that study, smoking half a pack a day doubled the formation of ss-DNA breaks in leukocytes, among other adverse effects. This was explained by the fact that cigarette smoke contains a large amount of substances that form reactive oxygen species by free radical-mediated reactions, which may increase the formation of ss-DNA breaks or reduce the capacity to repair these breaks [199].

A wide range of hormones and molecules that occur as a result of maternal oxidative stress are able to move to the cord blood, which is the peripheral blood of the fetus [200]. Another recent study by Chiba et al. demonstrated that maternal smoking causes fetal hypoxia that can lead to the production of reactive oxygen species. They found that a high oxidative stress in the mother also relates to a state of high oxidative stress in the neonates cord blood [201]. Moreover, previous studies have shown that the physical condition of neonates is affected by maternal stress, such as antioxidant concentration, reactive oxygen species, smoking, etc [202, 203]. All these data may support the correlation observed in our study of the shortening of infant telomeres due
to oxidative stress during maternal smoking in pregnancy. Villablanca et al. recently showed that neonatal arterial expression of pro-inflammatory genes which cause vascular injury is increased by prenatal and postnatal maternal exposure to environmental tobacco smoke [204]. This may also be a cause for the observed telomere shortening in infants with maternal exposure to tobacco smoke, as inflammation causes rapid turnover of blood cells (section 1.5.4.1).

The negative association between SJ maternal telomere length and smoking ever in pregnancy observed in our study approached significance (p = 0.057). There are contrasting results from different studies in the literature whereby some found a correlation between smoking and telomere length while others did not. For example, Morla et al. observed that smoking tobacco is significantly associated with telomere attrition in circulating lymphocytes of patients with or without chronic obstructive pulmonary disease (N = 76). They related this result to part of an “oxidant-induced senescence phenomenon” [205]. Another study (N = 1122) demonstrated a significant dose-dependent relation between telomere loss and smoking, where it was observed that each pack-year smoked per year was showing an additional 5 bp of telomere loss in comparison with the rate in their overall cohort of women [133].

In contrast, results from other groups showed no association between exposure to tobacco and telomere length. For example, Savale et al. compared patients with chronic pulmonary disease and control subjects and found no association (N = 291); additionally they observed no difference in telomere length between control smokers and non-smokers [206]. Another study by Ehrlenbach et al. observed no difference in relative telomere length between smokers, ex-smokers and non-smokers [207]. Our results for the SJ cohort would suggest that telomere attrition due to HIV or antiretroviral
In terms of the predictors considered in the regression model for the HIV/HAART-exposed group in the Pregnancy cohort, higher blood CD4$^+$ was found to be associated with shorter maternal telomere length. This may be explained by the decrease in CD4$^+$ cells induced by HIV infection, leading to increased turnover of activated CD4$^+$ (T helper) cells. Telomere length may limit the number of cell divisions that the lymphocytes undergo in response to antigens [208]. The T lymphocytes are derived from bone marrow progenitors which ultimately move and differentiate in the thymus. Naïve T lymphocytes are those CD4$^+$ and CD8$^+$ T cells that are present in peripheral blood and have not yet contacted foreign antigens. A decrease in naïve T lymphocytes occurs due to ageing of stem cells; it has been observed that haematopoietic stem cells undergo ageing and that telomere shortening is one of the mechanisms in limiting the self-renewal capacity of these cells [209]. When naïve T cells are exposed to an antigen, they are activated and become effector cells. Some of these cells later turn into long-lived memory T cells once the antigen is cleared [210].

An estimate of 15-20 cell divisions take place starting from a naïve cell to the expansion of effector cells during a normal immune response. Cell divisions occurring after that cause telomere shortening and ultimately replicative senescence [210]. Weng et al. found that memory CD4$^+$ T cells had shorter telomeres compared to naïve CD4$^+$ T lymphocytes [117]. These findings can help to explain our observation that increased CD4$^+$ count reflecting the normal differentiation of CD4$^+$ T lymphocytes from naïve to memory cells was associated with telomere shortening.

Longer duration of HIV infection at delivery was another variable that predicted short maternal telomere length in the HIV/HAART-exposed group of the Pregnancy
cohort. One may expect that longer HIV infection is related to longer exposure to antiretroviral drugs. However, in our cohorts, exposure to ART or HAART was not significantly associated with shorter telomere length. The shortening of maternal telomeres with greater duration of HIV infection is probably better explained by the rapid turnover of CD8\(^+\) T lymphocytes. The increased turnover of CD8\(^+\) T-cells reflects HIV-specific cytotoxic T lymphocyte response [24]. Chronic inflammation during HIV infection also increases white blood cell turnover [136] which may additionally be an explanation for telomere attrition with longer duration of HIV since whole blood contains 0.7% of white blood cells among which 15-40% are T lymphocytes [211].

In a similar linear regression analysis for the cord blood in our study, we observed that greater maternal age at birth was associated with longer telomeres. As mentioned in section 1.5.4.3, previous studies have found that sperm telomere length increases with age [150] and that there is a positive and significant correlation between paternal age and the off-springs' telomere lengths [151]. Since older mothers may have their child with older fathers, this may partly explain our observation. Cord blood contains stem cells and hematopoietic cells [212]. Stem cells express telomerase which could explain the maintenance of telomere length observed in cord blood. However, why an increase in cord telomere length was observed with increase in maternal plasma viral load and CD4\(^+\) count is unclear.

We investigated the correlation between several parameters. In exploring the association between maternal telomere length and maternal age, we found no correlation between the two for either the exposed or unexposed group, in either cohort. This is consistent with the observation by Rufer et al. that lymphocyte telomere length decreases rapidly with age during the first few years of life, then remains stable during adult years (roughly between 25-50 years of age) before declining again later in life.
The age range of the women in our study was 17 to 44 years, therefore it is not surprising that telomere length is fairly stable and does not show much attrition in our subjects.

When analyzing the association between maternal age and infant telomere length, a significant and positive correlation was found when considering all samples in the SJ cohort but not for the Pregnancy cohort. A possible explanation for the difference may lie in the discrepancy in ethnicity between the two cohorts. For example, the Pregnancy cohort did not have any Black-African or Hispanic participants in their unexposed control group whereas the SJ cohort did, in their ART-unexposed group. Moreover, the Pregnancy cohort counted mostly Caucasians while the majority of subjects in the SJ cohort were Black-Africans. In the literature, there is evidence for the role of ethnicity in telomere length. Recently, Roux et al. observed that blacks and Hispanics have shorter telomeres than whites (n=981). Additionally, the correlation of age with telomere length in women was six times greater in blacks and Hispanics than in whites [191].

Another study however reported contradictory evidence within a similar age group (n=1968) where they found that blacks had longer telomeres than whites [213]. In another small study, Okuda et al. saw no difference in telomere length between blacks, whites and Hispanics [192]. It has been suggested that the influence of race differences on telomere length may increase with age. Roux et al. suggested that, besides the heritability of telomeres, environmental factors may also play a role in ethnic differences [191]. Our study comprised mothers (and fathers) from different ethnic groups who may have been exposed to different environments. This can possibly explain the differences observed in the correlations between infant ATL and their mothers’ age in the two cohorts of our study.
When relating maternal ATL with that of their infant, we saw a weak positive but significant correlation for both groups in the Pregnancy cohort, something that was not seen in the SJ cohort. The Pregnancy cohort result is consistent with the literature whereby offspring telomere length is strongly related to paternal but is only weakly related so to maternal telomere length [148]. Unfortunately, within these cohorts, demographic information or clinical samples were not available for the fathers, something that has been changed since. There were a few outliers in the Pregnancy cohort. These may have contributed to driving the correlation. Ethnicity differences may also once again explain some of the differences seen here between the two cohorts.

As mentioned earlier, cord blood contains stem cells which would maintain their telomeres by expressing telomerase, unlike most other tissues of the body. This may explain the lack of significant correlation observed between cord blood ATL and infant blood ATL in the Pregnancy cohort. The cord blood is the infants’ side of the blood. Therefore, it may not be surprising to observe a similar correlation between cord blood ATL and maternal ATL as that seen between infant and maternal ATL. When relating cord blood ATL with maternal ATL in the Pregnancy cohort, we again saw no significant correlation. This was consistent with the result observed between maternal and infant ATL in the SJ cohort, but not in the Pregnancy cohort.

When both the cohorts were compared, it was observed that average maternal and infant ATL were quite different. DNA samples from dried blood spots in the SJ cohort consistently had longer telomeres than samples from whole blood in the Pregnancy cohort. Although the reason for this observation is not fully understood, a recent report has showed that HIV-1 DNA PCR testing of venous blood was 95% sensitive compared to 98.3% when performed on dried blood spots [214]. Moreover, the differences in ethnicities between the two cohorts may partly explain this observation.
Consistent with the study by Hunt et al [213] where they found longer telomeres in blacks compared to whites, the majority of our SJ cohort counted Black-Africans whereas there were none in the Pregnancy cohort.

Although the differences in ATL between the groups never reached statistical significance, we saw a pattern whereby samples that were exposed to HIV/ART or HIV/HAART, whether maternal, infant or cord blood, had consistently shorter relative ATL than unexposed ones. It should be noted that the variability observed among control infants exceeded what had been reported in the literature and had been used to guide our statistical plan. The power of our study was somewhat limited because of this. Whether statistically significant or not, the fact remains that the differences remains small (between 6% and 24% depending on the comparison). Their biological and clinical significance is difficult to ascertain at this stage.

The following table shows the percentage differences between the means of the two groups for infants and their mothers in both the cohorts.

Table 10: Differences between the means of ART/HAART-exposed and unexposed groups in the SJ and Pregnancy cohorts

<table>
<thead>
<tr>
<th></th>
<th>Unexposed</th>
<th>Exposed</th>
<th>% Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJ</td>
<td>7.9</td>
<td>7.8</td>
<td>-1.3</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>5.5</td>
<td>5.1</td>
<td>-7.2</td>
</tr>
<tr>
<td><strong>Mother</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJ</td>
<td>6.3</td>
<td>5.9</td>
<td>-6.3</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>3.5</td>
<td>3.4</td>
<td>-2.9</td>
</tr>
<tr>
<td><strong>Cord blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy</td>
<td>6.2</td>
<td>5.6</td>
<td>-9.7</td>
</tr>
</tbody>
</table>
3.2 Other limitations

So far, no studies have been carried out on the differences in telomere lengths in adults from ethnic groups other than blacks and whites. Therefore further investigations on the differences in telomere length between more varied ethnicities may be required to assess the effect of race on telomere length.

Considering the fact that telomere length is more strongly influenced by paternal inheritance, one major limitation to our study is that we did not have the fathers’ information. This would have allowed us to better explore the correlations and strengthen the results that we observed.

In the correlation between infant and maternal ATL for the Pregnancy cohort, there were a few outliers in both the HIV/HAART-exposed and unexposed control groups. These outliers mainly consisted of infants with relatively longer telomere lengths which may have been responsible for driving the positive correlation to significance. Therefore, the outliers may be one of the factors for the difference in infant-maternal ATL correlations observed between the SJ and the Pregnancy cohort.

In terms of the covariates taken into the model, smoking ever in pregnancy predicted shorter maternal telomere length for the SJ cohort. Although we did have some information about the timing of the smoking behavior, as most women who smoked ever smoked throughout their pregnancy, we did not distinguish between the two. However, we did not have any information about the quantity of smoking such that we could not distinguish between someone who smoked one cigarette a week versus a pack per day, nor could we differentiate a new smoker from a longtime one. This likely represents a major limitation in our study.
Another observation to the study which can be considered as a limitation is the varying telomere lengths with different types of blood sample collection. Dried blood spot was the sample that was collected from the SJ cohort whereas whole blood was used from the Pregnancy cohort. There may have been differences in the composition of the two types of samples that gave rise to the variations in the telomere length observed between the two cohorts. Even though it is unknown which type of blood sample is the ideal one, using the same sample collection protocol for all the samples would have been preferable and may have allowed larger comparison between the two cohorts. Additionally, for the SJ cohort, some mothers’ DBS samples were collected through direct finger pricks and some from blood collected by venipuncture that was then blotted onto the paper. We lacked information on that.

While blood was used in this study, it is known that different NRTIs affect different tissues [46]. Besides stem cells, germ line cells and tumor cells, telomerase is expressed in low levels in somatic tissues that require rapid division. Other than cells of the immune system which are present in blood, telomerase is expressed in the intestinal crypt cells [215], the basal layer of the skin [216] and hematopoietic progenitor cells [171]. Therefore, NRTIs may show greater effect on telomere shortening in the other tissues that express telomerase. Blood is the most easily available tissue, especially from newborns. Other tissues such as mouth swabs or hair follicles could also be investigated. Collecting solid organ tissues can require invasive procedures and physicians may be unwilling to perform and parents would be unlikely to consent to it.
3.3 Future directions

We observed a slight trend toward decreasing telomere length with increasing drug exposure when the unexposed, monotherapy and dual therapy groups were explored separately in the SJ cohort. To make a stronger link between telomere shortening and HAART exposure, it would be of interest to include patients on long-term HAART. Embryos/fetuses are exposed to the drugs through their mother for a short period of time. If HAART really did accelerate telomere shortening, it would be more prominent in patients who are on long-term therapy. Many women in our study were on HAART to prevent vertical transmission of the virus and not for their own health. Therefore their exposure was relatively short, as was the case for the infants. In the clinical setting, it is known that all NRTIs are not equally toxic in terms of side effects. It would be of interest to carry out investigations to study whether some drugs exert more effects than others on telomere attrition. This would help to discriminate which particular antiretroviral therapy combination and/or individual reverse transcriptase inhibitor (both NRTI and NNRTI analogues) is associated with accelerated telomere shortening. The antiretroviral drug/combination of drugs that has the least effect on telomere length may thus be preferred, especially for the treatment of pregnant women, infants and young children. Clinical studies are not easily feasible in the context of pregnancies or childhood, therefore studies with tissue culture systems would be of great use.

To test the relative toxicity of the drugs as well as the drug concentration effects, primary cell lines could be transfected with human telomerase and cultured with sub-lethal doses of antiretrovirals (that are recommended for use in pregnancy in developed and developing countries) alone and in combination. Since AZT has been shown to inhibit telomerase in vitro and in vivo, it could act as a positive control. Different drug
concentrations could be applied; even if a very high concentration causes cell death for some drugs, it will confirm the reproducibility of any small effects seen with the physiological concentrations. These investigations would help us explore the effects of long term exposure to antiretroviral drugs and the varying concentrations of these drugs that may significantly reduce the telomerase-dependent synthesis of telomere repeats.

Mouth epithelium (mouth swabs) have been obtained from newborns, therefore this can be used as one of the tissues other than blood for telomere length studies.

Although newer NRTIs are already available on the market that appear less toxic, little is known about their potential inhibitory activity toward telomerase. Furthermore, they are not commonly administered to HIV-infected pregnant women due to the lack of clinical trial data in this population. This is because pharmaceutical companies have little incentive to conduct new drug clinical trials with pregnant women considering the unknown risks of teratogenicity. Therefore AZT is still the first line NRTI used and it is felt that the slight risk to the infants with the current HAART regimen outweighs the greater risk of unforeseen toxicity. However, women are becoming pregnant on newer regimens already so studying all available drugs would be of great value.

3.4 Conclusions

With the use of HAART, the risk of mother to child transmission of HIV during pregnancy has been drastically reduced. With adequate antenatal care, less than 1% HIV transmission occurs. Inspite of this beneficial effect, NRTIs have the potential to affect the reverse transcriptase activity of the telomerase enzyme and hamper the maintenance of telomere length. This is of concern since telomere shortening has been associated with aging and many age-associated diseases. The access to antiretroviral
therapy has increased rapidly throughout the world; millions of HIV infected women, pregnant and non-pregnant and their children are exposed to HAART. This makes it important to increase the awareness of the possible long-term side effects of the therapy.

The goal of this thesis was to investigate telomere shortening in blood of infants and HIV-infected mothers who were exposed to ART/HAART during pregnancy and compare them with infants and mothers who were HIV/HAART unexposed (Pregnancy cohort) and HIV-exposed ART-unexposed. Quantitative PCR was the assay used to determine the average telomere length. Although the clinical study found no significant difference in the average telomere length between the two groups, several covariates taken considered in the model predicted shorter telomere length.

Because developing fetuses, infants and children are most susceptible to the pro-aging effects of the antiretroviral drugs, further investigations are warranted to gain a deeper understanding of the effect of these drugs on telomere maintenance.
References

62. Kühn, L., et al., Distinct risk factors for intrauterine and intrapartum human immunodeficiency virus transmission and consequences for disease progression in


