DYNAMICS OF TELOMERE LENGTH AND MITOCHONDRIAL DNA CONTENT IN A COHORT STUDY OF HIV-INFECTED AND HIV-UNINFECTED PREGNANT WOMEN AND CELL CULTURE MODELS

by

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Abstract

Globally, women constitute around 50% of HIV-infected individuals. While mother-to-child transmission accounts for 90% of new HIV infections among children, combination antiretroviral therapy (cART) reduces the risk from 25% to <2%. Treatment guidelines now promote lifelong cART for all persons living with HIV. This implies that more women will be conceiving on cART and exposing their unborn child over a longer period. Nucleoside reverse transcriptase inhibitors can have off target effects. These drugs can inhibit reverse transcriptase activity of telomerase, which could lead to a shortening of leukocyte telomere length (LTL). LTL has been described as a marker of cellular aging and a predictor of age-related diseases over time. Several antiretrovirals (ARVs) can exert mitochondrial toxicity, leading to mitochondrial dysfunction. The overarching hypothesis of my research was that LTL and mitochondrial DNA (mtDNA) content would be affected by cART in the clinical and cell culture samples.

I measured LTL in blood samples collected from 64 HIV-infected and 41 HIV-uninfected women at three visits during pregnancy using monochromatic multiplex quantitative polymerase chain reaction. CART treatment status during pregnancy was not associated with shorter LTL. However, smoking throughout pregnancy and receiving a boosted protease inhibitor regimen were independently associated with shorter LTL among HIV-infected women. Whether these reflect telomere attrition or redistribution of cellular subsets is unclear.

In clinical studies, it is challenging to distinguish between the effects of HIV *vs.* those of cART. I used cultured placental and T-lymphoblast cells to study the changes in mtDNA content following either short term exposure to individual ARVs at increasing concentrations, or longer-term exposure (21 days) to cART regimens at $1\times C_{max}$, (maximum concentration) prior to returning the cells to cART-free medium for ten more days, to allow recovery/repair. Most

ARVs and cART studied here induced increased mtDNA content, postulated to reflect mitochondria biogenesis in response to cellular stresses and/or damage, something that could promote the clonal expansion of mtDNA mutations. However, changes in mtDNA content in response to ARV exposure can be both bidirectional and cell-specific; and appear to be reversible. Mitochondria morphological changes were suggestive of increased mitophagy to preserve mitochondrial health.

Lay Summary

Approximately 90% of children living with HIV acquired their virus from their mother. Antiretroviral therapy (ART) with a cocktail of drugs has successfully reduced mother to child transmission from 25% to <2%. However, ART can also have unwanted effects on cells, such as affecting telomeres, the protective caps at the end of chromosomes, which shorten by each cell division. ART can also affect the DNA contained in mitochondria, the powerhouse of the cell. Within HIV-infected women, I found no evidence that ART is associated with shorter telomeres, except for one type of ART. However, smoking appeared to impact telomeres. I also detected that most ART treatments increased mitochondrial DNA content, likely in response to ART-induced cellular stress. This study showed that different drugs used by pregnant women can affect our cells in a way that could potentially have long term effects.

Preface

This dissertation is my original work and is based on the experiments conducted and designed by me in consultation with my supervisor, Dr. Hélène Côté and my supervisory committee. 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, H04-70540, and H07-03136). All study participants provided written informed consent.

The first chapter is an introduction to my thesis, beginning with a review of the relevant literature and finishing with the overarching hypothesis and main objectives of this study. In order to show an overview of HIV and pregnancy in Canada, I graphed the data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP) and made figures 1.2, 1.4, showing number of pregnancies per year and rate of pre-term delivery among women living with HIV in Canada between 1990 and 2016. Using the same set of data, I prepared figures 1.3, 1.5, and 1.6, illustrating changes over that same period with respect to the timing of ART initiation during pregnancy, as well as the make-up of cART regimens received during pregnancy. These figures were prepared by me, with CPHSP's permission to use the data and figures for my thesis. This permission was granted by Drs. Ari Bitnun and Laura Sauve via email. I also shared these figures with them.

A version of chapter two has been published as Anthony Y.Y. Hsieh, Sara Saberi, Abhinav Ajaykumar, Kyle Hukezalie, Izabella Gadawski, Beheroze Sattha, and Hélène C.F. Côté (2016), Optimization of a Relative Telomere Length Assay by Monochromatic Multiplex qPCR on the LightCycler 480: Sources of Variability and Quality Control Considerations, The Journal of Molecular Diagnostics, 18 (3):425-37. The permission to use these materials was

granted through Copyright Clearance Center (Confirmation Number: 11677467). I and A.Y.Y.H are the joint first authors and equally contributed to this work. Given that TL measurement is central to many biomedical research and epidemiology studies, we framed this study on the detailed optimization of a monochromatic multiplex qPCR (MMqPCR) method to more accurately and efficiently measure TL using the LightCycler 480. This technique was initially developed using a different instrument with many separate reagents. This work is a refinement and extension of the MMqPCR method previously published as DeAnna L. Zanet, Sara Saberi, Laura Oliveira, Beheroze Sattha, Izabella Gadawski, Hélène C. F. Côté (2013), Blood and Dried Blood Spot Telomere Length Measurement by qPCR: Assay Considerations, PLoS ONE, 8 (2): e57787. https://doi.org/10.1371/journal.pone.0057787. Initially, I optimized the MMqPCR using the LightCycler 480 and showed a strong correlation between MMqPCR and monoplex qPCR. However, we noticed an unacceptably high variability related to changes in the qPCR kits used over time, which prompted us to optimize the chemistry of the assay (initiated by S.S. and completed by A.Y.Y.H.) to accommodate a commercial one-component reaction mix and reduce the potential sources of variation. Finally, I validated the assay against other TL measurement methods, such as terminal restriction fragment, southern blot, flow fluorescent in situ hybridization, and monoplex qPCR. I have been involved in troubleshooting the assay throughout. I wrote the introduction and part of the method, result, and discussion. I did the statistical analyses and made figures 2.12 to 2.18. Figures 2.1 to 2.11 were generated by A.Y.Y.H. but in part based on my previous work. I critically reviewed and edited the final version of the manuscript. This method is now robust and high-throughput, and has been in use in our laboratory to measure TL for many studies, including the study in chapter three of this thesis.

Chapter three: “Dynamics of Leukocyte Telomere Length in HIV-infected and HIV-uninfected Pregnant Women: A Longitudinal Observational Study” is a manuscript ready to be submitted to a peer-reviewed journal. I am the first author of this manuscript co-authored by Steve E. Kalloger, Mayanne M. T. Zhu, Beheroze Sattha, Evelyn J. Maan, Julie van Schalkwyk, Deborah M. Money, Hélène C.F. Côté, and the CIHR Team in Cellular Aging and HIV Comorbidities in Women and Children (CARMA). I wrote the manuscript. For this study, I extracted all the demographic and clinical characteristics of the study participants from the CARMA-PREG database. I did all the cross-sectional univariate statistical analyses and was responsible for the interpretation of the multivariable longitudinal analysis which was done by Steve E. Kalloger.

Chapter four: “Effects of Pharmaceutical Concentrations of Antiretroviral Drugs on Mitochondrial DNA Content and Dynamics in Cell Culture Models” is a manuscript in preparation. I am the first author of this manuscript co-authored by Felix Valentino, Mayanne Zhu, Izabella Gadawski, Bryce Pasqualotto, Gordon Rintoul, Hélène C.F. Côté, and the CIHR Team in Cellular Aging and HIV Comorbidities in Women and Children (CARMA). I wrote the manuscript, participated in the design of the study, carried out all the cell culture experiments, collected the cells, extracted the DNA, and measured the mtDNA content of almost all the cell culture samples. I also performed the fluorescence microscopy with the assistance of Bryce Pasqualotto and Gordon Rintoul. Finally, I did the analysis, generated the figures, interpreted the results, and I am writing the manuscript.

Chapter five is the summary of my thesis, contributions and significance to the field, limitations of the studies, and future directions.

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List of Abbreviations

3TC	Lamivudine
AIDS	Acquired immune deficiency syndrome
ABC	Abacavir
AIDS	Acquired immune deficiency syndrome
ALB	Albumin
ART	Antiretroviral therapy
ARVs	Antiretrovirals
ATP	Adenosine triphosphate
ATV	Atazanavir
AZT	Zidovudine
cART	Combination antiretroviral therapy
CD4	Cluster of differentiation 4
CI	Confidence interval
CV	Coefficient of variation
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
DLV	Delavirdine
DNA	Deoxyribonucleic acid
DRV	Darunavir
DBS	Dried blood spot
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EVG	Elvitegravir
ETR	Etravirine
EVG	Elvitegravir
FDA	Food and Drug Administration
Flow-FISH	Fluorescent in-situ hybridization
FTC	Emtricitabine
GA	Gestational age
hTERT	Human telomerase reverse transcriptase
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
IC	Internal control
IDV	Indinavir
InSTIs	Integrase strand transfer inhibitors
kb	Kilobase
LT-IC	Long telomere internal control

MTCT	Mother to child transmission
mtDNA	Mitochondrial DNA
$\Delta\Psi$	Mitochondrial membrane potential
nDNA	Nuclear DNA
NFV	Nelfinavir
NRTIs/NtRTI	Nucleoside/nucleotide analog reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PI	Protease inhibitor
LPV/r	Ritonavir-boosted lopinavir
OXPHOS	Oxidative phosphorylation
PI/r	Ritonavir-boosted protease inhibitor
POLγ	Polymerase gamma
pVL	Plasma viral load
qPCR	Quantitative polymerase chain reaction
RAL	Raltegravir
ΔRFU	Relative fluorescence units
RPV	Rilpivirine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
RTV	Ritonavir
SGA	small for gestational age
SQV	Saquinavir
ST-IC	Short telomere internal control
STELA	Single telomere length analysis
SD	Standard deviation
TDF	Tenofovir disoproxil fumarate
TRF	Telomere Restriction Fragment
TL	Telomere length
C_T	Threshold cycle
UNAIDS	The Joint United Nations Programme on HIV/AIDS
WB	Whole Blood
WHO	World Health Organization

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Dedication

To My Parents

Chapter 1: Introduction

1.1 HIV/AIDS Epidemic

Since the beginning of the Human immunodeficiency virus (HIV) epidemic, 78 million people have become infected with the virus, and 35 million have died of HIV-related diseases. In 2016, 36.7 million people were living with HIV worldwide. According to Joint United Nations Programme on HIV/AIDS (UNAIDS), young people (15-24 years of age) represent a third of new HIV infections (UNAIDS, 2017). Women are vulnerable to HIV due to gender and physiological differences (UN Women, 2016). Approximately 50% of adults living with HIV are women, and HIV represents a leading cause of death among women of reproductive age. Worldwide, an estimated 2.1 million children are living with HIV, and 160,000 new HIV infections occurred among children in 2016 (UNAIDS, 2017).

Over the past two decades, combination antiretroviral therapy (cART) has drastically reduced the rate of HIV transmission and HIV-related morbidity and mortality. More than 18 million people living with HIV had access to cART in 2016, due to improved cART coverage. In 2016, 76% of HIV-infected pregnant women were receiving cART to prevent Mother to child transmission (MTCT) compared to 47% in 2010. As a result, new HIV infections among children have declined by 70% since 2001 (UNAIDS, 2017). In addition, 43% of children living with HIV accessed therapy in 2016, up from 17% in 2010. Nevertheless, much work remains to achieve access to cART for all who need it.

At the end of 2014, 75,500 Canadians were estimated to be living with HIV. This represents a 10% increase since 2011, which could be due to new HIV infections as well as fewer deaths due to successful ART (Public Health Agency of Canada, 2015). Men who have

sex with men continue to be the largest group (slightly above 50%) among people living with HIV in Canada. Injecting drugs (19%) and heterosexual sex (31%) are the other major risk factors for HIV acquisition in Canada. Women represent 22% of all people living with HIV in Canada. New vertical HIV infections are exceedingly rare due to high coverage of highly efficient and free of charge prevention of MTCT services in Canada (Public Health Agency of Canada, 2015).

1.2 HIV Pathophysiology

1.2.1 HIV

HIV is a lentivirus, a subgroup of the retrovirus family. The HIV genome contains two single-stranded RNA molecules. HIV primarily infects T-lymphocytes and macrophages expressing the glycoprotein CD4 on their surface (CD4+) cells. All CD4+ cells such as T helper cells, macrophages, dendritic cells, and astrocytes are susceptible to HIV (Leyssen *et al*., 2008). In the absence of treatment, HIV leads to a progressive destruction of the human immune system and causes acquired immune deficiency syndrome (AIDS) (Muesing *et al.*, 1985). Currently, HIV infection is a chronic rather than fatal disease due to the development of cART. However, there is no cure or effective vaccine for HIV prevention.

1.2.2 Progression of HIV Infection

The three phases of HIV infection are acute infection, chronic infection, and AIDS. Acute HIV infection is the earliest stage of HIV infection, which occurs shortly after HIV acquisition and usually lasts for two to four weeks. During this phase, the HIV RNA plasma viral load (pVL) increases rapidly by several orders of magnitude and viremia peaks in the human body. Between three to six weeks after HIV acquisition, antiviral immune responses can be detected (Ho *et al*., 1985; Cooper *et al.*, 1987), which are associated with a drastic decline in HIV pVL

and a temporary stabilization of the CD4+ cells. Acutely HIV-infected patients usually develop a viral syndrome, experiencing a severe depletion of central memory CD4+ T cells from the gut-associated lymphoid tissue (Guadalupe *et al*., 2003). It is hypothesized that the early injury to the gut immune system followed by subsequent damages to the gut epithelial cells induces gut permeability and the translocation of microbial products such as lipopolysaccharides. This microbial translocation then contributes to the systemic immune activation observed in persons living with HIV (Borrow *et al*., 1994). Following induction of immune responses, there is a chronic phase, also known as asymptomatic HIV infection. Throughout this latent period, HIV continues to actively multiply (Douek *et al*., 2003), and CD4+ cell count declines in a steady rate (Figure 1.1). This decline is not present or subtle in a small percentage of HIV-infected individuals, who are long-term non-progressors (Auger *et al*., 1988; Jones *et al*., 1992).

The final stage of HIV infection is AIDS. HIV-infected individuals are diagnosed with AIDS when their CD4+ cell count becomes lower than 200 cells/μl (normal CD4+ cell count is between 500-1,500 cells/μl), and their body increasingly cannot fight off HIV and opportunistic infections (Weber, 2001).

In summary, HIV RNA pVL and CD4+ cell count are the two markers of HIV disease progression. In general, higher HIV pVL is associated with faster decline in CD4+ cell count and disease progression (Macias *et al*., 2001; Goujard *et al*., 2006). Peak viral load on record is the highest recorded HIV pVL in the medical history of a patient, which is dependent on the timing of HIV testing (Ho *et al*., 1985; Cooper *et al*., 1987). HIV pVL drops to a relatively stable level within months to one year after infection, something known as “set-point” HIV pVL. The latter is indicative of immunological control and a low set point pVL correlates with slower disease progression (Mellors *et al*., 1996, 1997; Deeks *et al*., 2004). The CD4+ cell count is the most

important predictor of disease progression and of an individual's relative immune strength (Goujard *et al*., 2006; Phillips and Lundgren, 2006). The nadir CD4+ cell count is the lowest CD4+ cell count ever measured for an individual. It is a predictor of slower immune recovery, long-term morbidity, and increased risk of HIV-related and non-HIV-related illnesses among HIV-infected persons (Kaufmann *et al*., 2005).

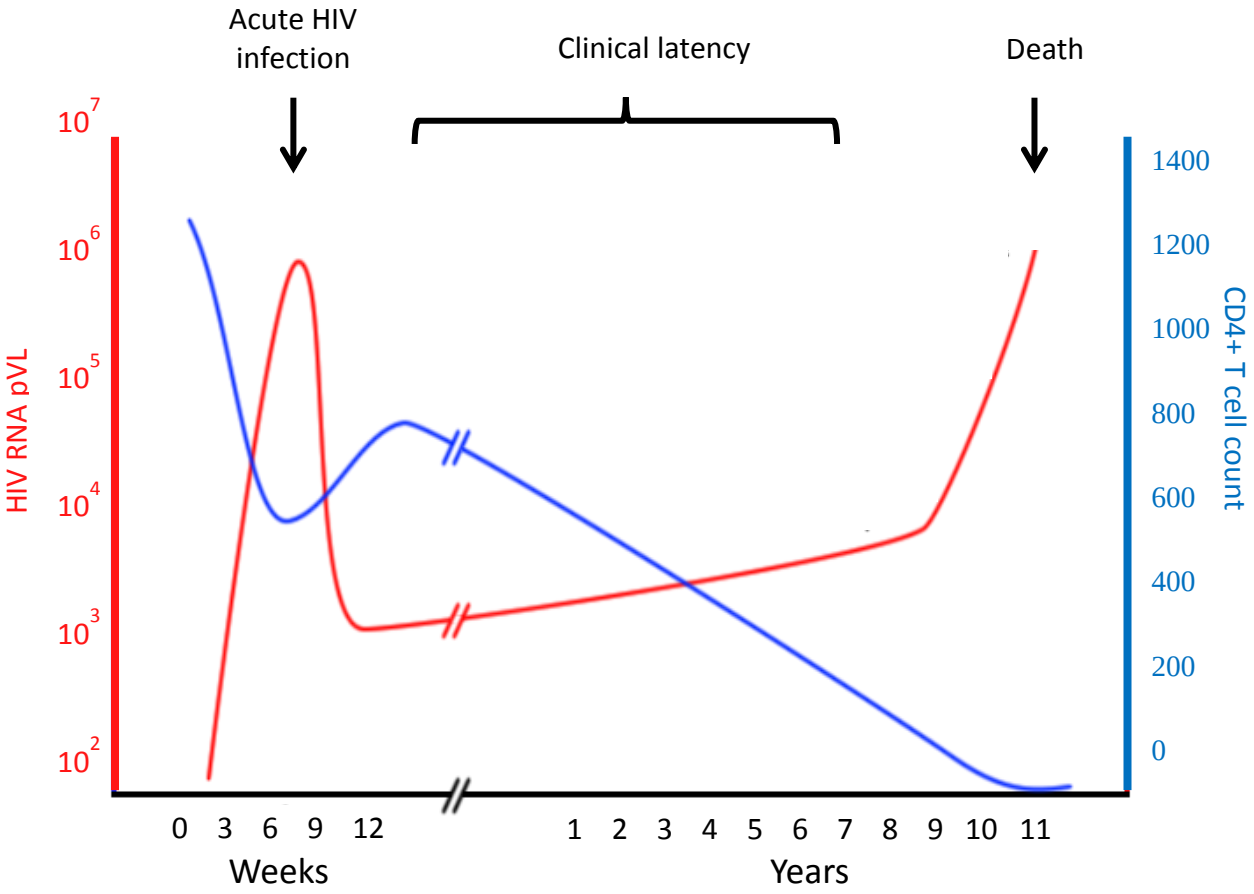

Figure 1.1. Dynamics of CD4+ cell count and HIV RNA pVL over the course of HIV infection. This figure was adapted from https://en.wikipedia.org/wiki/HIV#/media/File:Hiv-timecourse_copy.svg

1.2.3 HIV Replication Cycle

The HIV replication cycle is a multi-stage process, each stage crucial to successful replication of the virus, and therefore a potential target of antiretrovirals (ARVs). HIV begins its life cycle by binding (through the virion's glycoprotein 120) to co-receptors on the surface of CD4+ cells (Nakai and Goto, 1996). Once HIV fuses to a cell, it uses its reverse transcriptase (RT) to convert HIV RNA into double-stranded HIV DNA (Ferguson *et al*., 2002). Next, another viral enzyme called integrase inserts the HIV DNA into the host cell's DNA (Ferguson *et al*., 2002; Nielsen *et al*., 2005). The integrated HIV DNA is then transcribed by host machinery into new copies of viral RNA, which are translated into a long viral polypeptide. Once new viral genome and polypeptide are produced, they are assembled into new virions. During viral maturation, HIV protease cleaves the polypeptide into individual proteins. The final step of the viral life cycle is budding, whereby the new viral envelope forms, the virion changes structure, and becomes infectious (Swanstrom and Wills, 1997).

1.3 HIV Antiretroviral Therapy

HIV ARVs prevent HIV replication by targeting and blocking different stages of the HIV life cycle. To date, more than 30 ARVs have been approved by the Food and Drug Administration (FDA) and 30 of them are presented in Table 1.1. These drugs are classified into six classes based on their mechanism of action: (1) nucleoside/nucleotide analog reverse transcriptase inhibitors (NRTIs/NtRTIs), (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (3) protease inhibitors (PIs), (4) integrase strand transfer inhibitors (InSTIs), (5) fusion inhibitors, and (6) entry Inhibitors (Arts and Hazuda, 2012). The first antiretroviral agent, zidovudine (AZT) was licensed to treat HIV-infected individuals in 1987. A decline in disease

progression and death rate was seen among HIV-infected patients treated with AZT (Brook, 1987). The major challenges of AZT and other early NRTIs were inconvenient dosing, drug toxicities, and drug resistance due to incomplete virological suppression (Collier *et al*., 1996; D'Aquila *et al*., 1996). PIs and NNRTIs were introduced in the mid-1990s. In 1996, the introduction of the highly active antiretroviral therapy (HAART) revolutionized the management of HIV infection. HAART regimens usually consisting of two NRTIs plus a PI or NNRTI, effectively suppressed viremia and rapidly led to dramatic reductions in HIV-related morbidity and mortality in the developed world (Palella *et al*., 1998; Kaplan *et al*., 2000; Huang *et al*., 2006). The strategy of using a combination of three or more antiretrovirals from at least two different classes is still the standard of HIV treatment and is herein referred to as cART.

Table 1.1. Antiretroviral drugs (ARVs) approved by FDA for HIV treatment.

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)			
Brand Name	**Generic Name**	**Abbreviation**	**Approval date**
Retrovir	Zidovudine	AZT, ZDV	Mar-87
Videx	Didanosine	ddI	Oct-91
Hivid	Zalcitabine[1]	ddC	Jun-92
Zerit	Stavudine	d4T	Jun-94
Epivir	Lamivudine	3TC	Nov-95
Ziagen	Abacavir sulfate	ABC	Dec-98
Videx EC	Didanosine	ddI	Oct-00
Viread	Tenofovir disoproxil fumarate[2]	TDF	Oct-01
Emtriva	Emtricitabine	FTC	Jul-03
Vemlidy	Tenofovir alafenamide[3]	TAF	Nov-15

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
Brand Name	**Generic Name**	**Abbreviation**	**Approval date**
Viramune	Nevirapine	NVP	Jun-96
Rescriptor	Delavirdine	DLV	Apr-97
Sustiva	Efavirenz	EFV	Sep-98
Intelence	Etravirine	ETR	Jan-08
Edurant	Rilpivirine	RPV	May-11
Protease Inhibitors (PIs)			
Brand Name	**Generic Name**	**Abbreviation**	**Approval date**
Invirase	Saquinavir mesylate	SQV	Dec-95
Norvir	Ritonavir	RTV	Mar-96
Crixivan	Indinavir	IDV	Mar-96
Viracept	Nelfinavir mesylate	NFV	Mar-97
Agenerase	Amprenavir	APV	Apr-99
Kaletra	Ritonavir boosted Lopinavir	LPV/r	Sep-00
Reyataz	Atazanavir sulfate	ATAZ-ATV-TA2	Jun-03
Lexiva	Fosamprenavir calcium	FOS-APV	Oct-03
Aptivus	Tipranavir	TPV	Jun-05
Prezista	Darunavir	DRV	Jun-06
Integrase Strand Transfer Inhibitors (InSTIs)			
Brand Name	**Generic Name**	**Abbreviation**	**Approval date**
Isentress	Raltegravir	RAL	Oct-07
Tivicay	Dolutegravir	DTG	Aug-13
Vitekta	Elvitegravir	EVG	Sep-14
Fusion Inhibitors			
Brand Name	**Generic Name**	**Abbreviation**	**Approval date**
Fuzeon	Enfuvirtide	T-20	Mar-03
Entry Inhibitors			
Brand Name	**Generic Name**	**Abbreviation**	**Approval date**
Selzentry	Maraviroc	MVC	Aug-07

[1]no longer available as of December 31, 2006. [2]Tenofovir disoproxil fumarate, an acyclic phosphonate is a nucleotide analog reverse transcriptase inhibitor (NtRTI). [3]TAF was approved by the FDA in 2015 as part of a coformulation that also includes elvitegravir, the pharmacokinetic enhancer cobicistat, and emtricitabine. Since then it has been approved as part of other coformulations; it is not available as a single agent.

1.3.1 Nucleoside/nucleotide Analog Reverse Transcriptase Inhibitors (NRTIs/NtRTIs)

NRTIs/NtRTIs were the first class of FDA-approved ARVs for HIV treatment (Young, 1988). NRTIs are administered as prodrugs which need to be phosphorylated intracellularly to their active triphosphate derivatives (Furman *et al*., 1986). NRTIs are nucleoside analogs of adenosine, thymidine, guanosine, or cytidine, which structurally mimic substrate nucleotides of the HIV RT, but lack a 3′-hydroxyl group on the deoxyribose. Once the NRTIs are activated to their triphosphate form, they target HIV RT and inhibit the conversion of HIV RNA to DNA through competition with the natural deoxynucleotides, leading to early chain termination (Cheng *et al*., 1987; Richman, 2001). Although they play a crucial role in HIV therapy, NRTIs can also induce side effects. Mitochondrial DNA (mtDNA) polymerase gamma (POLγ) can be inhibited by NRTIs, which can lead to mtDNA depletion or mutation and subsequent mitochondrial dysfunction (Lim and Copeland, 2001; Lewis *et al.*, 2003, Yamanaka *et al.*, 2007). Mitochondrial toxicity can result in moderate to life-threatening toxicities, such as lipodystrophy, neuropathy, lactic acidosis, and hepatotoxicity (Zaera *et al*., 2001; Côté *et al*., 2002; Nolan *et al*., 2003; Kohler and Lewis, 2007), and this was especially true with the earlier generations of NRTIs. In addition, NRTIs are also known to inhibit human telomerase reverse transcriptase (hTERT) and, in *in vitro,* studies have been shown to lead to progressive shortening of telomeres (Strahl and Blackburn, 1996; Hukezalie *et al*., 2012; Leeansyah *et al*., 2013).

1.3.2 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs inhibit HIV RT through conformation changes, induced by the binding of the NNRTIs to the HIV RT. Unlike NRTIs, NNRTIs do not require intracellular metabolism to be in their active form (Kohlstaedt *et al.*, 1992). Currently, there are five FDA-approved NRTIs, including efavirenz (EFV), nevirapine (NVP), delavirdine (DLV), etravirine (ETR), and

rilpivirine (RPV). NNRTIs have tremendously contributed to HIV therapy; however, use of NNRTIs has been associated with drug toxicities (Rivero *et al*., 2007). A relationship between NVP use in pregnancy and hepatotoxicity has been confirmed by a number of studies (Lyons *et al*., 2006; Natarajan *et al*., 2007; van Schalkwyk *et al*., 2008). EFV is the more favourable NNRTI in many countries as it is less hepatotoxic than NVP and does not require dose adjustment (Chersich *et al*., 2006). However, it has neuropsychiatric effects, including emotional instability, insomnia, impaired concentration and abnormal dreams (Nachega *et al*., 2007). According to FDA retrospective data, maternal EFV treatment during pregnancy was associated with neural tube defects, and other more severe birth defects in animal models (Schwetz, 2002). For this reason, EFV was avoided during pregnancy for a long time. However, a study by Copp *et al*. (2013), has not revealed any significant increase in birth defects in HIV-infected women exposed to EFV during pregnancy, irrespective of trimester of exposure. Since 2013, the WHO has recommended EFV-based cART, a combination of tenofovir (TDF) + lamivudine (3TC) or emtricitabine (FTC) + EFV, as the first-line treatment for pregnant and breastfeeding women (WHO, 2013) Despite this, there are still concerns regarding EFV safety during pregnancy, especially during first trimester for which additional data is needed (Pillay and Black, 2012; Ford *et al*., 2014). More recent data suggested that TDF/FTC/EFV was associated with a lower risk for adverse birth outcomes than NVP and LPV/r based regimens (Zash *et al.*, 2017).

1.3.3 Protease Inhibitors (PIs)

PIs target the active site of HIV protease, the enzyme responsible for cleavage of the viral protein precursors during virion maturation (Weber and Agniswamy, 2009). The advent of PIs had a striking impact in the history of ART, although the high pill burden of early PIs affected adherence, hence long-term viral suppression (Danner *et al*., 1995). Although PIs are generally

well tolerated, they can also induce adverse effects, such as hepatotoxicity, nephrotoxicity, cardiotoxicity, and metabolic abnormalities (Hughes *et al*., 2011). Saquinavir (SQV) was the first PI approved by FDA, followed by ritonavir (RTV). RTV was not well tolerated due to several side effects including severe gastrointestinal symptoms, paresthesia, elevated hepatic aminotransferases and serum triglyceride levels (Cameron *et al*., 1998; Notermans *et al*., 1998) but it reduced the metabolism of other PIs through hepatic and intestinal cytochrome P450 3A4 inhibition. It is now used as a "booster" in PI formulations, to increases the level of other PIs in the plasma (Kempf *et al*., 1997). Nelfinavir (NFV) was once used extensively in pregnancy. However, after contamination of drug stocks (released prior to March 31, 2008) with the teratogenic chemical ethyl methane sulfonate, and highly variable drug dose, and therefore the risk for subtherapeutic concentrations during pregnancy (Hirt *et al*., 2006), it is no longer used. Ritonavir-boosted PI (PI/r) regimens have been extensively used in pregnancy in the developed world, and as a second line treatment in the developing world. Several studies have reported increased rates of pre-term birth in women treated with PI-based regimens (Grosch-Woerner *et al.*, 2088; Powis *et al.*, 2011; Chen *et al*., 2012; Sibiude *et al*., 2012, Yonatan *et al.*, 2016). The mechanism behind this association remains unclear. Possible effects of RTV on the maternal-fetal adrenal system have been suggested as a potential reason (Sibiude *et al*., 2012).

1.3.4 Integrase Strand Transfer Inhibitors (InSTIs)

InSTIs prevent the integration of viral DNA into host DNA by inhibiting the integrase enzyme involved in the strand transfer (Espeseth *et al*., 2000). Raltegravir (RAL) was the first InSTI approved by the FDA in 2007 (Temesgen and Siraj, 2008). In 2012, the next InSTI, elvitegravir (EVG) was approved as part of a fixed-dose daily tablet containing TDF, FTC, and the cytochrome P450 isoenzyme 3A inhibitor cobicistat (Temesgen, 2012). Dolutegravir (DTG)

is the newest InSTI and its main advantage over prior InSTIs is that it can be given once daily without boosting (Rathbun *et al*., 2014). It has been shown that RAL has a high antiviral potency in both ART-naive and -treated patients (Temesgen and Siraj, 2008; Eron *et al*., 2013). Currently, cART usually contains a backbone of two NRTIs plus a third agent (base): a NNRTI or a PI/r or an InSTI (DHHS, 2016; WHO, 2016). The benefits of RAL-based regimens in rapid viral suppression has prompted some clinicians to prescribe this drug to treat HIV-infected pregnant women presenting late in pregnancy, to control their pVL before delivery and prevent vertical transmission (Boucoiran *et al.*, 2015). US guidelines have also suggested the use of RAL in these situations. Nevertheless, data remains limited for RAL in pregnancy and it should be further investigated.

1.3.5 Entry and Fusion Inhibitors

Entry and fusion inhibitors block receptors at the cell surface and thereby interfere with the binding, fusion, and entry of HIV into host cell (Qian *et al*., 2009). No birth defects were reported in 36 cases of exposure to enfuvirtide, a fusion inhibitor and 18 cases of exposure to maraviroc, an entry inhibitor during pregnancy. However, data are insufficient to be evaluated statistically and allow one to make comments on the efficacy and safety of these drugs in pregnancy (Vogler, 2014).

1.4 HIV and Pregnancy

Since the beginning of the HIV epidemic, millions of women have acquired HIV, primarily through heterosexual contact. Furthermore, as mentioned earlier, the vast majority of pediatric HIV infections occur through vertical transmission from mother to child. In the absence of any intervention, the overall risk of vertical transmission is 15-30% in non-breastfeeding populations and 20−45% in breastfeeding population (De Cock *et al*., 2000;). In 1994, the

Pediatric AIDS Clinical Trials Group protocol 076 (PACTG 076) demonstrated that zidovudine (AZT) monotherapy in pregnancy reduced the risk of MTCT by 67.5% (from 25.5% to 8.3%) (Connor *et al*., 1994). Initially, AZT monotherapy was given orally during pregnancy, followed by an intravenous dose during labour. Prophylactic AZT was also given orally to the newborn for the first six weeks of life. This strategy was the standard of care for HIV-infected pregnant women until the late 1990's. According to a large prospective cohort study published in 2002, MTCT rate for women receiving AZT monotherapy was 10.4% *vs.* 3.8% for those receiving dual therapy and 1.2% for those receiving a combination of three or more ARVs (Cooper *et al*., 2002).

The first WHO guidelines on the use of ART for the prevention of MTCT were published in 2001 and 2004. Historically, guidelines for ART initiation in pregnancy were based on WHO staging or CD4+ cell count for non-pregnant adults. In 2010, the CD4+ threshold for ART initiation was raised from ≤200 cells/µL to ≤350 cells/µL (WHO, 2010). Then in 2013, the WHO guidelines recommended cART for all HIV-infected pregnant women regardless of CD4+ cell count or WHO stage, and continuation of ART for life (WHO, 2013). It has been suggested that rate of MTCT may be lowest in infants born to mothers receiving cART prior to conception and continued throughout pregnancy (Townsend *et al*., 2014). However, there are limited data on the practical implementation and benefits of this strategy in pregnancy and on the potential toxicities and long-term risks of exposing developing embryo to maternal cART in the first trimester of gestation (Gopalappa *et al*., 2014; Mofenson and Watts, 2014). Approximately a quarter of all 75,500 Canadians living with HIV are women and the majority of them (75%) are of reproductive age (Public Health Agency of Canada, 2014). HIV-infected people now live longer, with a healthier reproductive life, leading to increasing number of pregnancies among women

living with HIV (Forbes *et al*., 2012; Caprara *et al*., 2014). Figure 1.2 shows the number of pregnancies per year among women living with HIV in Canada, from less than 50 per year in the early 1990's to approximately 200 per year in recent years.

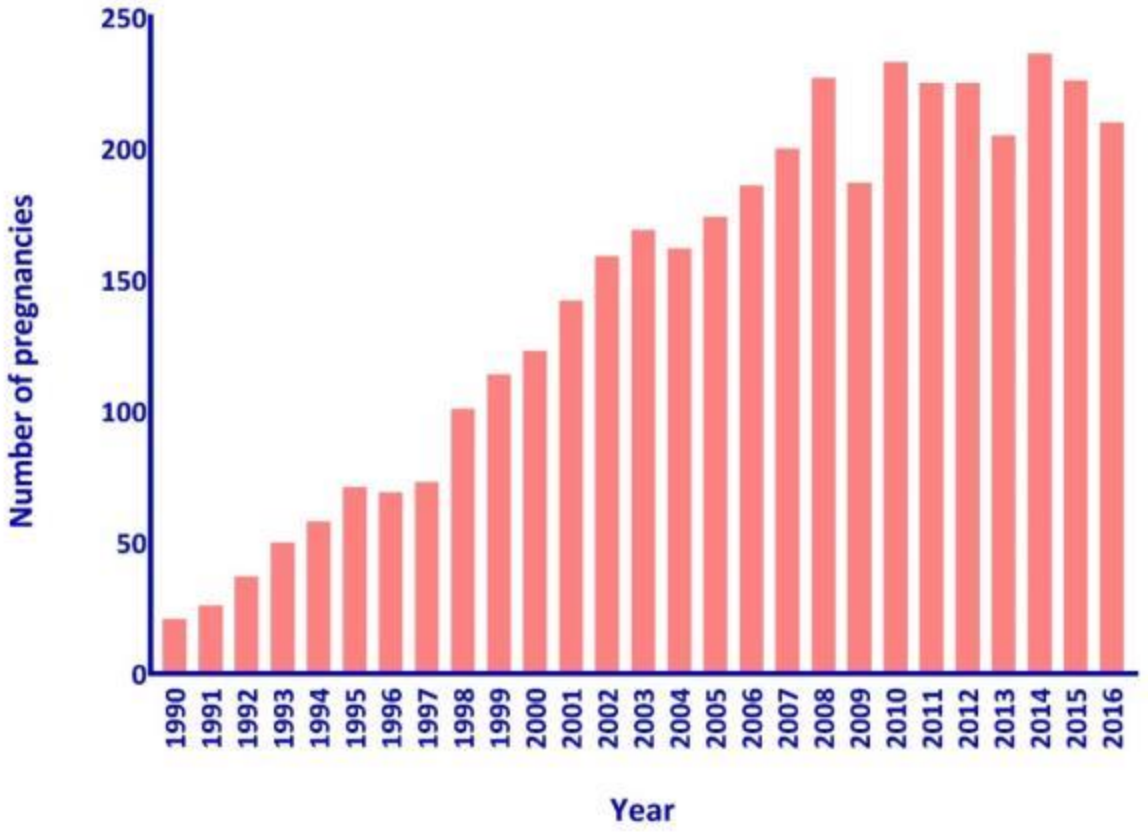

Figure 1.2. Number of pregnancies per year among women living with HIV in Canada between years 1990 and 2016. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email.

According to a national review (Forbes *et al*., 2012), rates of vertical transmission in Canada have been decreasing dramatically due to successful MTCT prevention services. Importantly, the rate of transmission between 1990 and 2010 was only 0.4% when cART regimens were initiated for mothers at least four weeks before delivery (Forbes *et al*., 2012). Figure 1.3 illustrates the timing of initiation of ART during pregnancy for women living with HIV in Canada between years 1990-2016. This shows that the proportion of women conceiving while on cART has been increasing steadily over time, to reach more than 60% in 2016.

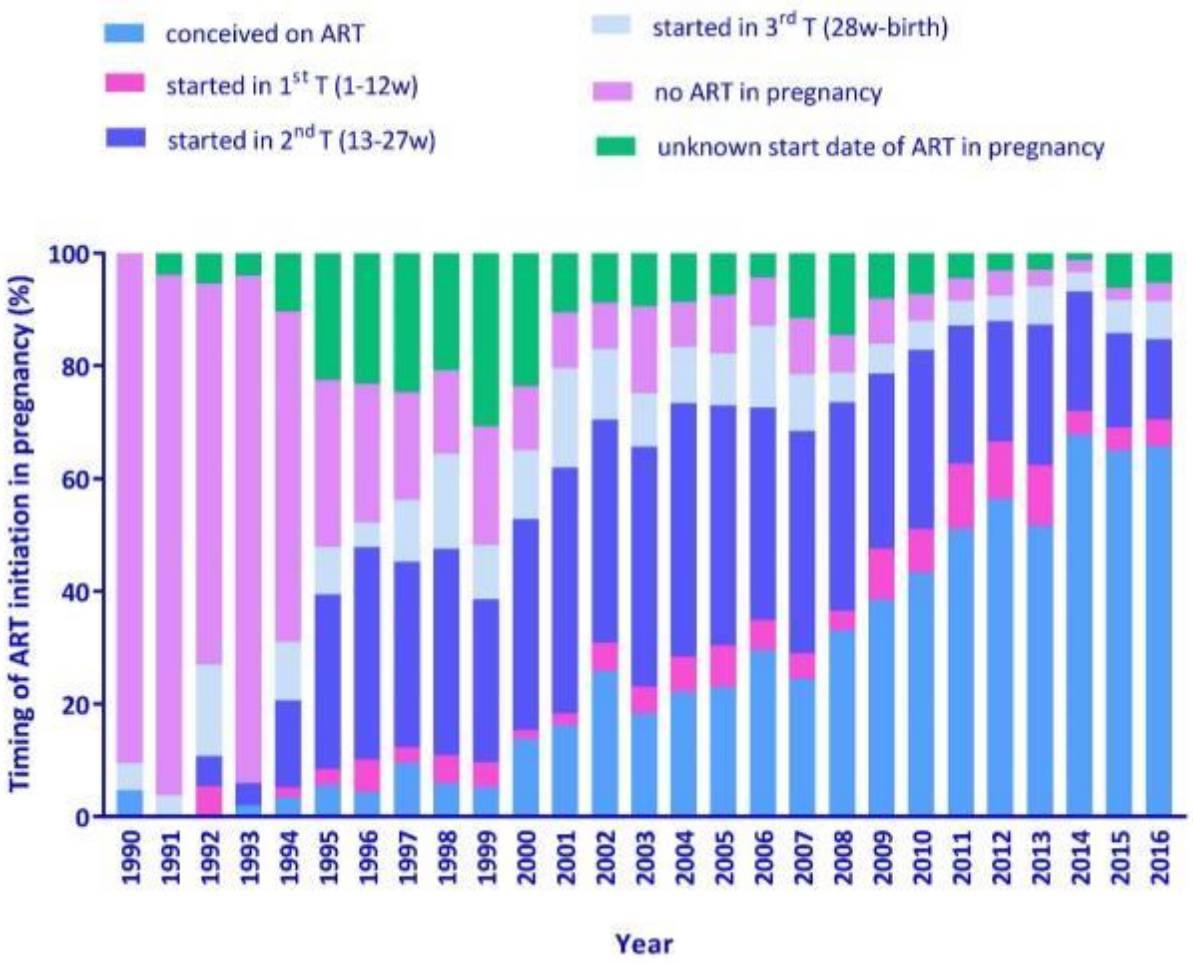

Figure 1.3. Timing of ART initiation in pregnancy for women living with HIV in Canada between 1990 and 2016. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email.

In Canada, all pregnant women living with HIV should be treated with cART, irrespective of their baseline CD4+ and pVL (Money *et al*., 2014). The latest guidelines published in 2014 recommended that cART regimens containing two NRTIs and a PI/r should be favored in the treatment of HIV-infected women because of higher confidence regarding their safety and efficacy during pregnancy. Moreover, ARVs which are known to cross through the placenta should be used in pregnancy whenever it is possible (Money *et al*., 2014). Although

there is an increasing body of research to guide the use of ARV during pregnancy, there are many newer agents developed in recent years for which such data are not yet available.

1.5 Outcome of Pregnancies with HIV Infection

There is a growing body of research addressing pregnancy outcomes among HIV-infected women. The results vary depending on the study population, location, medication access and adherence, and immune function. While many studies report an increased risk of adverse pregnancy outcomes, including pre-term birth (<37 weeks of gestation), growth restriction, preeclampsia and gestational diabetes (Thorne *et al*. 2004; Suy *et al.*, 2006; Townsend *et al*., 2007; Gonzalez-Tome *et al*., 2008; Powis *et al*., 2011; Chen *et al*., 2012; Sibiude *et al*., 2012; Watts *et al*., 2013), other studies reported no difference between HIV-infected and HIV-uninfected control women (Szyld *et al*., 2006; Patel *et al*., 2010; Rudin *et al*., 2011; Boyajian *et al*, 2012).

For example, the association between pre-term birth and use of PIs in pregnancy still is the subject of debates. Several studies reported an increased risk of pre-term deliveries in HIV-infected women who received PIs in pregnancy (Thorne *et al*., 2004; Townsend *et al*., 2007). While some studies indicated an association between pre-term birth and PIs in general, other studies implicated the possible role of ritonavir boosting as a risk factor for pre-term birth (Powis *et al*., 2011; Sibiude *et al*., 2012; Chen *et al*., 2012). A recent Canadian study reported an increased risk of pre-term birth among HIV-infected pregnant women who received PI/r (19.3%) *vs.* unboosted PI (10.8%) regimens during pregnancy (Kakkar *et al*., 2015). This finding is consistent with a large observational study from Botswana, reporting higher rates of pre-term birth among women treated with LPV/r during their pregnancy (Zash *et al*., 2017).

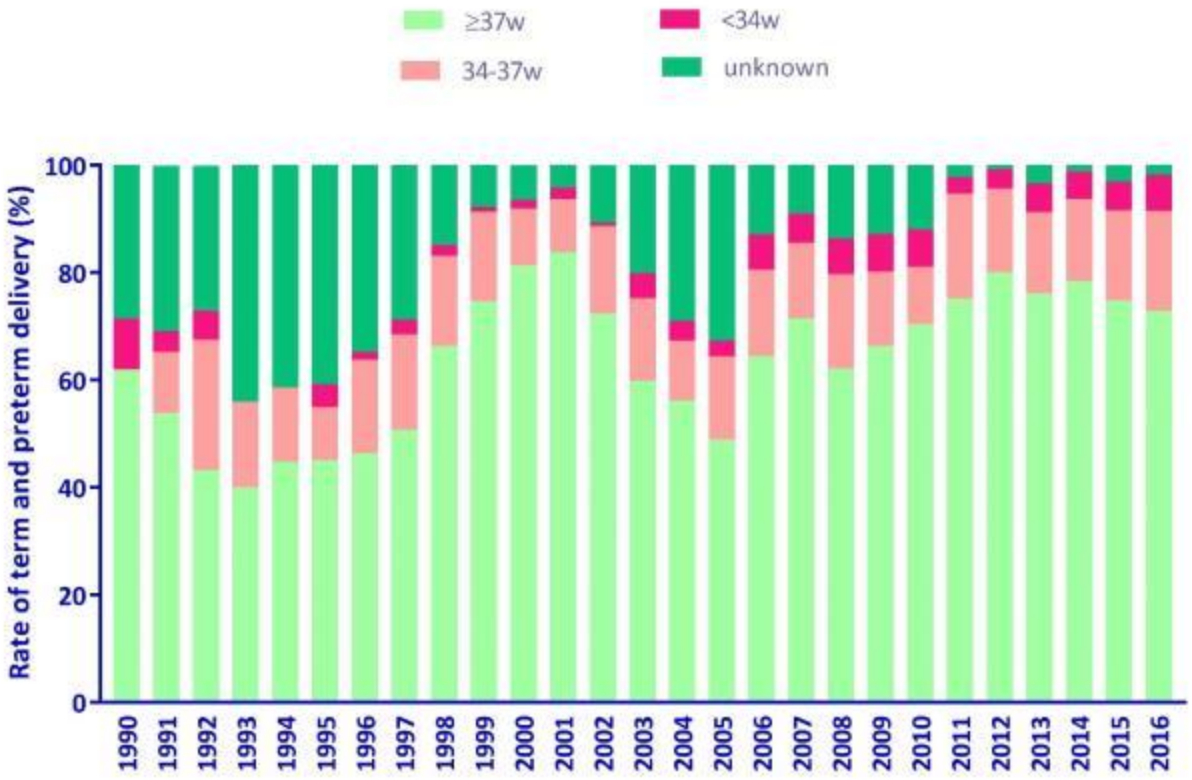

Figure 1.4. Rate of pre-term delivery among women living with HIV in Canada between 1990 and 2016; full term delivery: ≥37 weeks of gestation, pre-term delivery: between 34-37 weeks of gestation, very pre-term delivery:< 34 weeks of gestation. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email.

Figure1.4 illustrates the rate of pre-term birth among women living with HIV in Canada. It is not possible to define the overall rate of pre-term birth over the years due to a substantial proportion of pregnancies with unknown weeks of gestations. However, the average rate of pre-term birth was close to 25% between 2011 and 2016, when gestation time data was most complete. It is important to note that more than 50% of HIV-infected pregnant women were treated with PI/r during their pregnancy between 2008 and 2015. Of note, the use of InSTIs in pregnancy has increased in recent years and 2016, more than 20% of pregnant women living with HIV in Canada received an InSTI (Figure1.5).

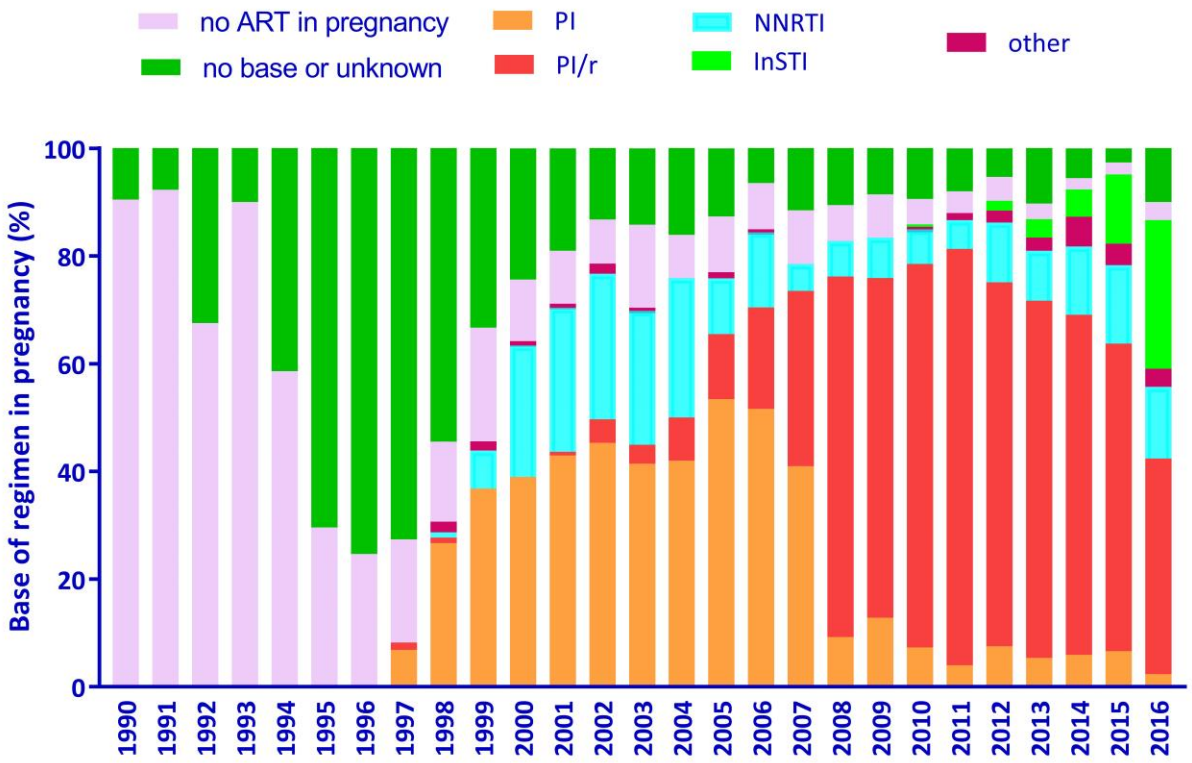

Figure 1.5. Base of cART regimens received in pregnancy by women living with HIV in Canada between 1990 and 2016. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email.

In summary, adverse pregnancy outcomes may be associated with HIV infection, specific antiretroviral drugs, the timing of ART initiation in pregnancy, and the presence of confounding factors, such as ethnicity, smoking, and alcohol and illicit drug use (Kennedy *et al*., 2017). Previous studies have suggested links between placenta mitochondrial abnormalities and some pregnancy complications such as preeclampsia (Jauniaux *et al*., 2006; Mandò et *al.*, 2014). Similarly, telomere length (TL) and cell senescence have been implicated in pre-term deliveries and fertility (Hallows *et al*., 2012).

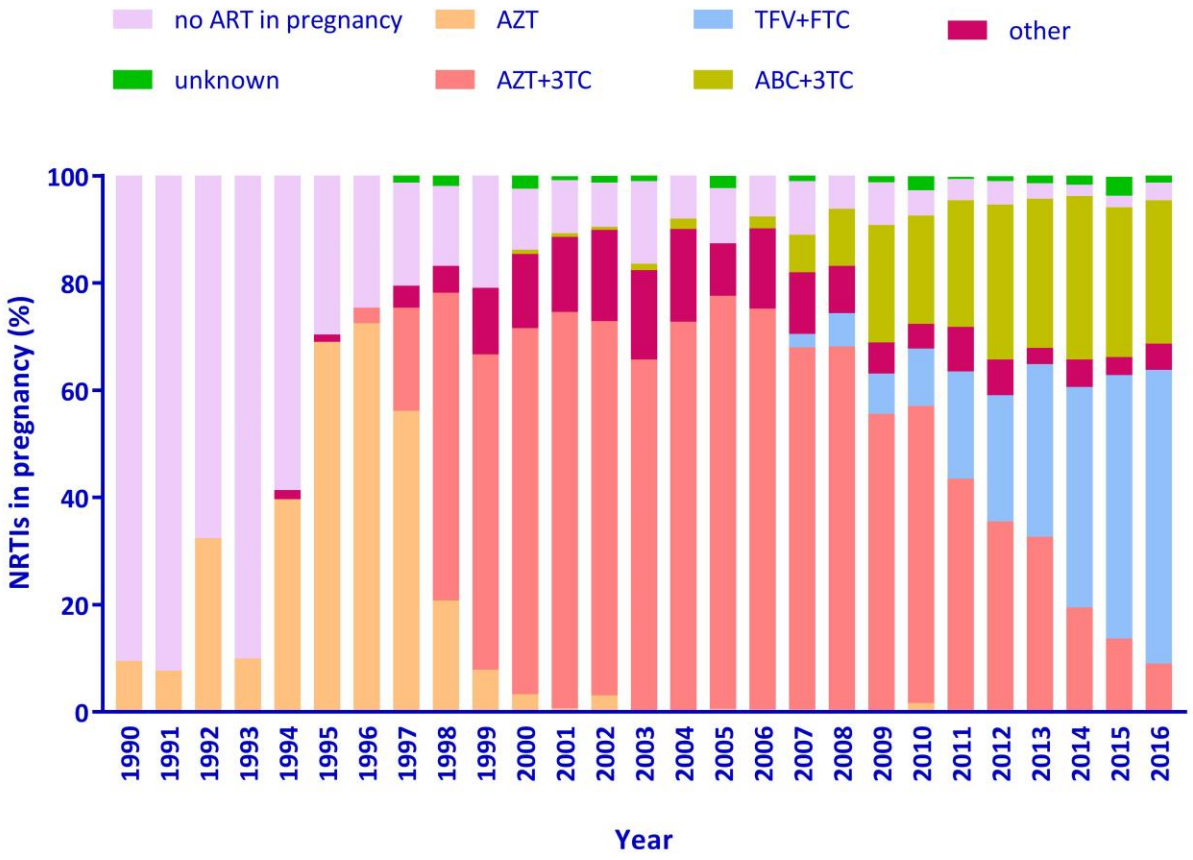

Figure 1.6. NRTIs received in pregnancy by women living with HIV in Canada between 1990 and 2016. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email.

1.6 Theories of Cellular Aging

HIV-infected people live longer due to the widespread use of cART. However, despite the great success of cART, people living with HIV develop age-related diseases, such as cardiovascular diseases, diabetes, malignancy, cardiovascular disease, bone disease, and osteoporosis earlier in life (Guaraldi *et al*., 2011; Deeks, 2011). These studies led to the hypothesis that HIV-infected ART-treated populations experience premature and/or accelerated aging. An emerging challenge is to understand how HIV-infected patients are aging. Aging can be defined as the "*time-related deterioration of the physiological functions necessary for survival and fertility*" (Gilbert, 2000). Two important theories that explain the aging process are telomerase inhibition/telomere shortening (Harley *et al*., 1992; Allsopp *et al*., 1992) and the oxidative stress theory of aging (Harman, 1956; 1981). HIV and/or cART can directly or indirectly contribute to aging through both of these pathways.

1.6.1 Telomerase Inhibition and Telomere Shortening, HIV, and cART

Telomeres are nucleoprotein sequences of TTAGGG (5–20 kilobases long) at the ends of the eukaryotic chromosomes (Moyzis *et al.*, 1988) that protect their ends from deterioration or fusion (Harley *et al.*, 1990). TL is maintained via the enzyme telomerase (Morin, 1989). Telomerase is a ribonucleoprotein consisting of two subunits: an RNA template (TERC) and a reverse transcriptase (TERT), which is essential for maintaining TL (Torres and Lewis, 2014). In somatic cells, telomeres shorten with each cellular division, as DNA-dependent DNA polymerases fail to replicate chromosomal ends (Vaziri *et al*., 1994). Progressive shortening of telomeres by cell division eventually causes replicative senescence when a critical telomere length is reached (Kim *et al*., 1994).

The rate of TL attrition can vary from one individual to another, depending on various factors, including stress, sex, socioeconomic status, education, and environmental factors such as cigarette smoking and alcohol consumption (Vasa-Nicotera *et al*., 2005; Valdes *et al*., 2005; Mayer *et al*., 2006; Möller *et al*., 2009; Puterman and Epel, 2012; Mezzinler *et al*., 2013; Rode *et al.*, 2015). NRTIs, including AZT, ddI, ABC, and TDF have been shown to inhibit telomerase activity in *in vitro*, leading to accelerated shortening of TL (Strahl and Blackburn, 1996; Datta *et al*., 2006; Liu *et al*., 2007; Hukezalie *et al*., 2012). The mechanism of this TL shortening is believed to involve inhibition of TERT via chain termination, in a manner similar to the mechanism of inhibition of the HIV RT (Peng *et al*., 2001). HIV infection itself can cause chronic immune activation, oxidative stress, and inflammation (Pace and Leaf, 1995; Appay *et al*., 2011; Deeks, 2011). In the absence of any NRTIs, HIV infection has been associated with reduced telomerase activity in activated CD4+ T-cells (Franzese *et al*., 2007) but in HIV-infected individuals, both increase and decrease telomerase activity have been reported in different T-cell subsets (Palmer *et al*., 1997; Lichterfeld *et al*., 2008). Several cohort studies, including one from our laboratory have reported shorter leukocyte TL in HIV-infected participants compared to HIV-uninfected controls (Pathai *et al*., 2013; Zanet *et al*., 2014; Lu *et al*., 2015; Auld *et al*., 2016). Although the mechanism behind this has not been fully elucidated, in addition to increased immune activation, it has been speculated that HIV may cause telomere shortening by decreasing telomerase activity within hematopoietic progenitors (Vignoli *et al*., 1998). In addition, it has been suggested that the oxidative stress associated with HIV chronic inflammation could contribute to accelerated telomere shortening (Von Zglinicki, 2002). Due to observed association between HIV infection, HIV therapy, and telomere shortening, there is a

need to investigate these relationships in HIV-infected population and in pregnancy, given that long term data remain scarce on the effects of cART exposure.

1.6.2 Oxidative Stress Theory of Aging, Mitochondria Toxicity, HIV, and cART

Although several pathways are considered to contribute to aging, mitochondria are well accepted as being central to the aging process (Loeb *et al*., 2005). In fact, their dysfunction was suggested as a pre-requisite for the aging process (Haendeler *et al*., 2009). Mitochondria are organelles essential to cellular metabolism in eukaryotic cells (Chinnery and Schon, 2003). They are responsible for energy production through the formation of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (Trifunovic and Larsson, 2008). Mitochondria have a double-membrane structure, consisting of internal and external membranes separated by an intermembrane space. The internal membrane is a folded structure, the mitochondrial cristae, and it contains the enzyme complexes of the mitochondrial respiratory chain. The external membrane is permeable to many solutes and allows the interchange of molecules with the cytosol (Dyall *et al*., 2004). Mitochondria are the only animal cellular organelles containing their own DNA, the mitochondrial DNA (mtDNA). MtDNA is a 16.6 kb double-stranded circular DNA, which encodes 13 proteins involved in the respiratory chain, two mitochondrial ribosomal RNA and 22 mitochondrial transfer RNA that are essential for the translation of mitochondrial-encoded proteins (Andrews *et al*., 1999). Unlike nuclear DNA, mtDNA is maternally inherited. Depending on the cell type and stage of development, cellular mtDNA content varies widely (Anderson *et al*., 1981). Tissues with higher oxidative metabolism contain more mitochondria and higher mtDNA content (Bratic and Trifunovic, 2010). Accordingly, higher mitochondrial activity might be required in special physiological states with high energy demand, such as fertilization and pregnancy (Morén *et al*., 2014).

In most individuals, the vast majority of mtDNA molecules share the same sequence (homoplasmy), whereas mutants often coexist with wild-type molecules (heteroplasmy) (Holt *et al*., 1988). Mitochondria play a central role in the aging process since they are both a major reactive oxygen species (ROS) producer, and a target of ROS. The proximity of mtDNA to the ROS-generating electron transport chain makes mtDNA susceptible to oxidative damage (Harman, 1972). The free radical theory of aging has been extended to become the mitochondrial oxidative theory of aging (Wohlgemuth *et al*., 2014). Since mtDNA encodes vital components of the OXPHOS and protein synthesis machinery, oxidative damage-induced mtDNA mutations that impair either the assembly or the function of the respiratory chain will, in turn, trigger further accumulation of ROS, which results in a vicious cycle leading to energy depletion in the cell and ultimately cell death (Fleming *et al.*, 1982). Additionally, mtDNA is replicated by POLγ, which has a lower fidelity than nuclear polymerase (Longley *et al.*, 2001). Therefore, mtDNA naturally carries a higher mutation rate than nuclear DNA. The accumulation of mtDNA damage over time, leading to mitochondrial dysfunction, forms the basis of the mitochondrial theory of aging (Muftuoglu *et al.*, 2014).

Mitochondria are highly dynamic organelles, which actively fuse, divide, propagate, and diminish depending on the cellular requirements. Mitochondrial morphology can be indicative of mitochondrial health, protein quality control, and cell survival (Bess *et al.*, 2012; Shutt and McBride, 2013). The mitochondria of aged individuals have been demonstrated to often be swollen with disrupted mitochondrial network and fewer cristae (Sastreet *et al.*, 2000), something also seen observed in kidney tissue following acute ARV-related mitochondrial toxicity (Côté *et al.*, 2006). Mitochondrial function, especially respiration and ATP production has been shown to decline with age and is considered as an important mediator of senescence

(Desler *et al*., 2012). Energy deficiency can eventually cause a wide range of metabolic and degenerative diseases (Wallace *et al.*, 2010). Alterations in structure and function of mitochondria have often been seen in age-related disorders such as Parkinson's disease, sarcopenia and metabolic diseases, including diabetes mellitus (Desler *et al*., 2012; Galloway and Yoon, 2012).

Mitochondrial fusion, fission, and autophagy are essential for mitochondrial maintenance, specifically in protection against persistent mtDNA damage (Chen *et al*., 2011; Bess *et al*., 2012). Therefore, changes in mitochondria morphology and mass might serve as a compensatory mechanism to maintain mitochondrial functions. For example, increased mitochondrial biogenesis, might also be an attempt to increase functional capacity of mitochondria under pressure (Lee and Wei, 2005). On the other hand, mitochondrial autophagy may exert have a protective mechanism against EFV-induced respiratory chain malfunction (Apostolova *et al.*, 2011). Hence, mitochondrial DNA quantity and quality are major contributors to mitochondrial functionality and may act as marker of mitochondrial health. As mentioned earlier, NRTIs, the backbone of cART, have been shown to inhibit mitochondrial POL γ leading to mtDNA depletion and mitochondrial dysfunction (Chen *et al.*, 1991). NRTIs can also inhibit hTERT activity in *in vitro* models, leading to accelerated shortening of telomeres (Leeansyah *et al*., 2013; Hukezalie *et al*., 2012).

In addition to its nuclear function in telomere maintenance, hTERT contains a mitochondria signal sequence and translocates to the mitochondria under increased stress conditions, where it protects against oxidative stress, DNA damage and apoptosis (Santos *et al.*, 2004; Saretzki, 2014). As this function appears dependent on the RT activity of hTERT (Sharma *et al.*, 2012), it could presumably be affected by NRTI inhibition. A recent study demonstrated

an association between ART-induced mitochondrial toxicity and increased mRNA expression of telomerase (Paintsil *et al*., 2014). Mitochondrial toxicity in the context of pregnancy can potentially lead to the physiological alterations and irreversible changes in fetal development (Morén *et al*., 2014). Indeed, further studies are needed to compare cART regimens, especially those used in pregnancy, with respect to their effects on telomere/telomerase and on mitochondria, as these could influence long-term health.

1.7 Research Hypothesis and Objectives

Shorter leukocyte TL (LTL) and mtDNA alterations may exert long-term effects on women and children in the absence of clinical symptoms. Optimizing the safety and efficacy of cART regimens during pregnancy requires the investigation of such subclinical alterations. In the context of clinical studies, it is challenging to distinguish between the effects of HIV infection itself *vs.* those of cART. The overall goal of my PhD project was to investigate the effects of cART at the cellular level in both clinical samples and cell culture models. The overarching hypothesis of my research was that LTL and mtDNA content would be affected by cART. My objectives were to:

1) measure LTL in HIV-infected pregnant women treated with cART and HIV-uninfected pregnant control women, and determine whether LTL is influenced by pregnancy, HIV, or cART.
2) measure mtDNA content in cell culture models (placental and T-lymphoblast cells) following treatment with various ARVs alone or in combinations and investigate the effects of either a) short term exposure to individual ARVs at increasing concentrations; or b) longer-term exposure (21 days) to cART regimens at pharmacological concentrations

Given that accurate and reliable measurement of TL is central to this study, the second chapter of this thesis describes the optimization of a monochromatic multiplex qPCR assay I needed for my LTL measurement. I then used this assay in chapter 3 to accomplish my first objective.

Chapter 2: OPTIMIZATION OF A RELATIVE TELOMERE LENGTH ASSAY BY MONOCHROMATIC MULTIPLEX QPCR ON THE LIGHTCYCLER 480: SOURCES OF VARIABILITY AND QUALITY CONTROL CONSIDERATIONS

2.1 Introduction

Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes. In humans, telomeres are non-coding double-stranded nucleotide repeats (TTAGGG)n, approximately 9-15 kilobases (kb) in length, that preserve chromosome stability and integrity (Moyzis *et al.*, 1988). In somatic cells, telomeres shorten with each cell division. The progressive shortening of telomeres to a critical length ultimately leads to cell senescence, affecting health and lifespan (Nilsson *et al.*, 2013). TL shortening has been associated with aging over long periods of time and with oxidative stress (Weischer *et al.*, 2012; kim *et al.*, 2016), and has been described in the literature as a marker of cellular replicative potential (Zhu *et al.*, 2011). In particular, shorter LTL is associated with increased risk of atherosclerosis and cardiovascular disease (Farzaneh-Far *et al.*, 2008). Consequently, there is great interest in reliably measuring TL in different biomedical research applications. However, the concept of measuring TL beyond the research arena, as part of routine clinical care or commercial tests remains controversial. This is partly because inherent inter-individual variability and a lack of standardization in its measurement limit the usefulness of TL and hinder its possible addition as a useful diagnostic tool. Despite this, some contend that clinical relevance of TL will improve as more data is collected, and may in the future have a role in a multifactorial health assessment (Wolinsky,

2011). For example, there are consistent data supporting the association between short LTL and cardiovascular health. Data from the Framingham Heart Study show associations between shorter LTL and systemic oxidative stress, increased carotid intimal medial thickness in obese men, higher renin to aldosterone ratio, and lower left ventricular mass (Demissie *et al.*, 2006; O'Donnell *et al.*, 2008; Vasan *et al.*, 2008; Vasan *et al.*, 2009). Similarly, LTL is negatively associated with the ASSIGN cardiovascular risk score (Lu *et al.*, 2015) , while the Framingham risk score is associated with the proportion of leukocytes with telomeres shorter than 5 kb (Fyhrquist *et al.*, 2011). The implementation of TL measurements in the standard of care will necessitate the development of high-throughput techniques with low variability and cost.

Several techniques are currently used to measure TL. Among them, terminal restriction fragment (TRF) measurement by traditional Southern blot is widely used. However, TRFs include subtelomeric DNA that can vary in length between individuals depending on the particular restriction enzyme used, introducing inter-individual variation independent of true TL. TRF measurements require large amounts of DNA (0.5–5 µg/individual) and long processing time, with the lack of standardization hindering inter-study comparisons (Aubert *et al.*, 2012). Flow-fluorescence in situ hybridization (Flow-FISH) is another widely used assay capable of measuring TL in subsets of cells (Baerlocher *et al.*, 2006). While this capability presents some advantage, Flow-FISH assays require a minimum of ~1-2 mL of fresh blood and cannot be used on solid tissues or archived samples (Baerlocher *et al.*, 2006; Wieser *et al.*, 2006; Aubert *et al.*, 2012). Furthermore, both TRF and Flow-FISH methods are labour-intensive (Lauzon *et al.*, 2000), restricting their feasibility in large-scale studies.

In 2002, Cawthon established a high-throughput qPCR-based method using partially mismatched primers to amplify the tandem repeats of telomeric DNA. In this monoplex assay,

the telomere fluorescent signals (T) of experimental DNA are quantified in one plate and normalized to the signal of a single copy nuclear gene (S) in another plate to generate a T/S ratio proportional to the average TL. This assay can measure relative TL in a small amount of DNA (10 ng) and in archived specimens (Cawthon, 2002). In 2009, a monochrome multiplex qPCR (MMqPCR) strategy was developed that determines the relative quantity of two amplicons with greatly different copy numbers and melting temperatures. The cycle thresholds (C_T) for the first and more abundant target (T) are collected at earlier cycles, when the signal for the less abundant target signal (S) is still at baseline (Cawthon, 2009).

Considering the growing interest in measuring TL in biomedical research, clinical diagnostics, and commercial applications, accurate and high-throughput methods are in demand. As of November 2015, 514 and 146 publications in the PubMed Central® archive have cited Cawthon's 2002 and 2009 publications, respectively. The advent of the MMqPCR would have been expected to end the use of the monoplex method as MMqPCR is categorically superior to the monoplex assay. It features lower variability (Aubert *et al.*, 2012), lower cost, improved concordance with alternate techniques (Cawthon, 2009), and requires lower amounts of sample material. Measured T/S ratios are virtually independent from pipetting volume, thereby reducing operator-induced variability. We believe that an impediment to the broader adoption of the MMqPCR methodology is rooted in the challenges encountered by many laboratories who tried to implement this assay with their instruments and reagents. Other confounders such as different qPCR instruments, standard calibration, single copy gene used, and assay variability may also exacerbate this problem.

The initial MMqPCR assay published used the Bio-Rad MyiQ Single Color Real-Time PCR Detection System (Cawthon, 2009). We initially attempted to use the same conditions on

the Roche LightCycler 480, another qPCR platform widely used in both research and clinical settings. However, dissimilarities in instrument performance, commercial reagents, and workflow required substantial optimization. Herein, this process is described in detail, as this may fulfil a need in the laboratory/research community, and promote the adoption of the MMqPCR TL assay. In doing so, we discuss general sources of variability and quality control criteria that should apply to MMqPCR users irrespective of their platform.

2.2 Materials and Methods

2.2.1 Study Specimens

Human cord blood (n=33), placenta (n=25), and Dried Blood Spot (DBS) (n=58) specimens were randomly selected from previously published studies (Imam *et al.*, 2012; Edmonds *et al.*, 2015). In addition, cultured human colorectal adenocarcinoma cells HT-29 (ATCC® HTB-38TM) treated with ARVs (ddI, d4T, AZT, TDF) (n=36) from a previous study were used (Hukezalie *et al.*, 2012).

2.2.2 DNA Extraction and Storage

Whole blood (WB) total genomic DNA were extracted using the QIAcube and QIAamp DNA Mini kit (Qiagen) according to the manufacturer's Blood and Body Fluid Protocol with the following modifications: 100 µL of WB was diluted in 100 µL of 1X phosphate-buffered saline, and DNA was eluted in 100 µL of Buffer AE (Qiagen), consisting of 10 mM Tris-Cl, 0.5 mM Ethylenediaminetetraacetic acid (EDTA), pH 9.0. Extracted DNA from blood was stored in AE buffer at -80°C until used. Genomic DNA from harvested HT-29 cells were extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and resuspended in 200 µl Elution Buffer (Qiagen). DNA from HT-29 cells were stored in Elution Buffer (Qiagen) at -20°C until used.

DBS total DNA was extracted using the Qiagen Dried Blood Spot protocol with the following modifications: DNA was extracted from six 3 mm diameter paper punch discs obtained from a single blood spot for each DBS. In the automated QIACube extraction protocol, 350 µL of buffer ATL, 20 µL of Proteinase K solution, 300 µL of buffer AL, and 150 µL of 95% ethanol were used. DBS DNA was eluted in 50 µL of buffer AE at stored at -80°C until used.

2.2.3 Quantitative PCR

Relative TL was expressed as a ratio between the quantity of telomeric DNA (T) normalized to the copy number of a single copy nuclear gene (S), yielding the T/S ratio. Two qPCR assays were used: a monochrome monoplex two-tube method and a monochrome multiplex one-tube method, referred to hereafter as MMqPCR. The monoplex assay was performed as described (Cawthon, 2002) with previously established modifications (Zanet *et al.*, 2014). Polymerase (DNA directed), gamma 2, accessory subunit (POLG2), and albumin were used as the single copy nuclear gene in the monoplex and MMqPCR assays, respectively. All primers were purified by the manufacturer using high performance liquid chromatography, and primer sequences are presented in Table 2.1.

Table 2.1. Primer Sequences.

Primer ID	Assay Type	Nucleotide Sequence*
ASPG3F	Monoplex	5'- GAGCTGTTGACGGAAAGGAG -3'
ASPG4R	Monoplex	5'- CAGAAGAGAATCCCGGCTAAG -3'
Tel1b	Monoplex	5'- CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT -3'
Tel2b	Monoplex	5'- GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT -3'
AlbuF	MMqPCR	5'- cggcggcgggcggcgcgggctgggcggAAATGCTGCACAGAATCCTTG -3'
AlbdR	MMqPCR	5'- gcccggcccgccgcgcccgtcccgccgGAAAAGCATGGTCGCCTGTT -3'
TelgF	MMqPCR	5'- ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT -3'
TelcR	MMqPCR	5'- TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA -3'

*Upper-case nucleotides represent genome complimentary regions, and lower-case nucleotides represent non-complimentary GC-clamps. Telomere, Tel; Albumin, Alb

The multiplex assay was carried out starting from a previously modified existing assay (Cawthon, 2009; Zanet *et al.*, 2013). For each MMqPCR PCR reaction, 8 µL of master mix and 2 µL of DNA (~20 ng/µL) were added to wells of a 96-well plate (Roche, Ref 04729692001) in duplicate. The final concentrations of reagents in the master mix were 1X LightCycler® SYBR Green I Master (Roche), 1.2 mM EDTA (Sigma-Aldrich), and 0.9 µM of each of the four MMqPCR primers (Table 2.1). Prior to PCR, the plates were centrifuged at 1500 X g for 2 min at room temperature. The qPCR was performed on a LightCycler® 480 using the following thermal cycling profile and ramping temperature rates (Figure 2.1):

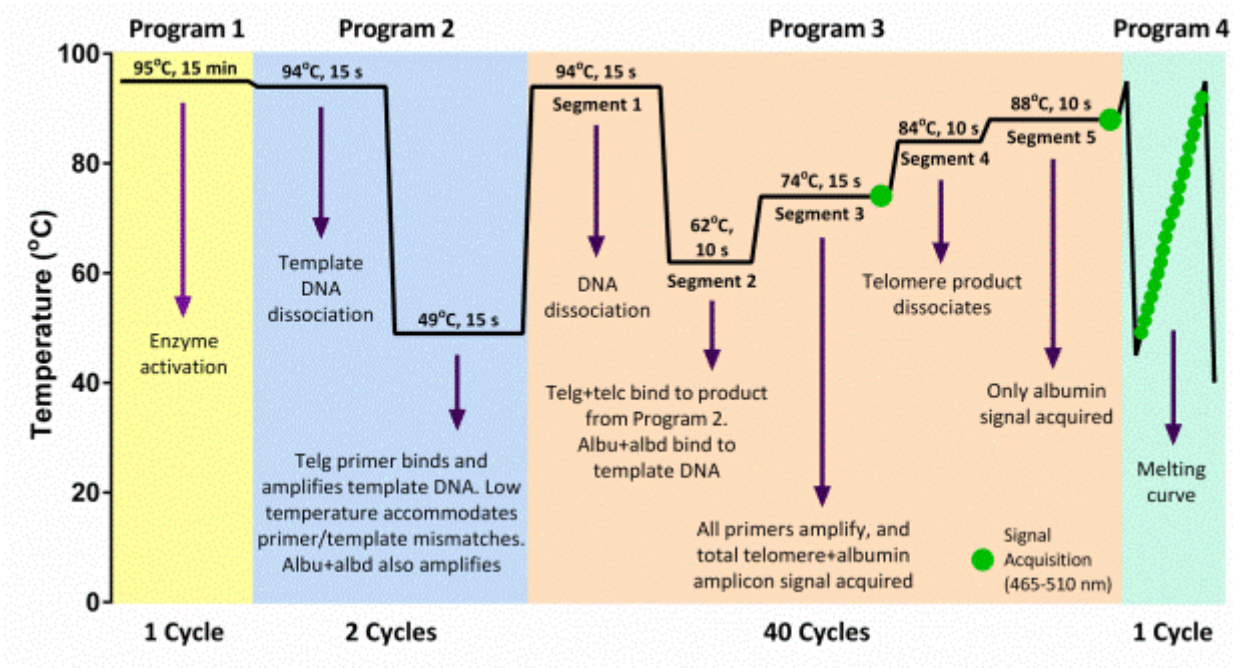

Figure 2.1. Schematic of the MMqPCR cycling profile, describing each stage. Program and Segment markers as referenced on Lightcycler 480 raw output are shown. The circles represent fluorescence signal acquisitions

The thermal cycling profile was initiated with a 95°C enzyme activation ("hot-start") incubation for 15 min. This was followed by 2 cycles of 94°C for 15 s (2.2°C/s), and 49°C for 15 s (2.2°C/s), then 40 cycles of 94°C for 15 s, 62°C for 10 s (2.2°C/s), 74°C for 15 s, 84°C for 10 s, and 88°C for 15 s, with signal acquisitions at the end of the 74°C and 88°C stages. After cycling, a melting curve program was run starting with a 95°C incubation for 1 min, followed by continuous acquisitions every 0.2°C for 45-95°C (ramping at 0.11°C/s). All temperature ramping rates were set at 4.4°C/s or 2.2°C/s where indicated, except the melting curve which was ramping at 0.11°C/s. Data was acquired using the LightCycler® 480 Software Version 1.5.1.62 SP2. Each run included a single standard curve, a negative control, and two positive internal controls (IC).

A standard curve was generated by serial dilution (1:2) of WB pooled from 24 healthy individuals, ranging from ~21 to 0.16 ng/µL of DNA across 8 standards resulting in a 128-fold

linear range (R^2>0.99). This range can be extended on the upper end, but was not necessary for our applications. Standard curves were included in a centre column of each plate to avoid plate edge effects. The T/S ratios were calibrated using a coefficient based on samples (n=35) that were assayed using both MMqPCR and Flow-FISH (Imam *et al.*, 2012), such that the leukocyte T/S ratio obtained with MMqPCR approximated the mean lymphocyte TL in (kb) determined by Flow-FISH. Measurements were carried out in duplicates for both samples and ICs, allowing a total of 40 samples per run.

Because the LightCycler instrument software does not currently permit dual signal acquisition processing, data were exported in text format then imported into Microsoft Excel to separate the 74°C acquisition data from 88°C acquisition data by sorting the Program and Segment markers in the LightCycler 480 Software output (Figure 2.1). The 74°C acquisition was defined as Program 3 Segment 3, and the 88°C acquisition was Program 3 Segment 5. Acquisition-delineated data was then converted to grid format in LC480Conversion Version 2.0, a free software from the Heart Failure Research Centre (HFRC) in Amsterdam, Netherlands and available online at (http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LC480Conversion.zip&description=LC480Conversion:%20conversion%20of%20raw%20data%20from%20LC480&sub=LC480Conversion). Baseline corrections and threshold cycle (C_T) calculations were obtained using LinRegPCR Version 2012.1 (HFRC) as previously described (Ruijter *et al.*, 2009). The software establishes a baseline and aligns the exponential phase of the fluorescence curve for all samples to produce a common “window of linearity”. The C_T is then defined as one cycle below the upper bound of the window of linearity to ensure that the fluorescence threshold is reached during exponential amplification. Individual sample efficiencies generated by LinRegPCR were

not used here. Instead, the PCR efficiency for each acquisition was calculated from the standard curve included on each plate.

2.2.4 Alternate TL Assays (TRF and Flow-FISH)

Mean TRF lengths from human placentae and cultured HT-29 cells were measured as previously described (Wong and Collins, 2006) (Hukezalie *et al.*, 2012). Median TL from cord blood lymphocytes and granulocytes were determined using Flow-FISH as previously described (Baerlocher *et al.*, 2006). Placenta, HT-29 cells, and cord blood were used as a matter of convenience, since the TRF and Flow-FISH assays had already been performed on these specimens.

2.2.5 MMqPCR Quality Control

In each run, a long telomere IC (LT-IC), a short telomere IC (ST-IC), and a no template negative control consisting of AE buffer were included. The LT-IC consisted of pooled WB DNA extracts, and ST-IC was DNA extracted from cultured cells (K562). IC T/S ratios, PCR efficiencies, % difference between duplicates were logged for each run. Apart from the requirement that the negative control must be negative or extremely low (>$3C_T$ below the lowest standard) and the fact that all collected data must lie within the bounds of the standard curve, quality control (QC) for this assay was established around five criteria:

The T/S ratio of the LT-IC must be within mean ± 2 standard deviations (SD) of logged values from previous runs done under the same conditions.

The T/S ratio of the ST-IC must be within mean ± 2 SD of logged values from previous runs done under the same conditions.

Based on the standard curve, the PCR efficiencies for both the 74ºC and the 88ºC acquisitions must individually lie between 90-100% (between 1.80 and 2.00-fold amplification per cycle).

The difference between the two PCR efficiencies must be <2.5% (or <0.05-fold amplification per cycle).

The mean of the absolute difference in T/S ratio between duplicates [=abs (value 1-value 2) *100/ (mean of values 1 and 2)] for all 40 samples in a run must be <10%.

Informally, if one of the IC moved in the same direction for >7 consecutive runs, the assay was halted for investigation of the instrument. To avoid rejecting an excessive percentage of runs, a run was accepted if it met at least four of the five QC criteria. Within an accepted run, individual samples for which duplicate T/S values varied by >15% between them (as per the equation described in the 5th QC criterion) were repeated in duplicate. If the repeat duplicates still differed by >15%, these were averaged with data from the first set of duplicates, and their value accepted if the four replicates showed a coefficient of variation (CV, SD*100%/mean) <10%. Any sample that failed all the above conditions was rejected permanently and excluded from further analyses.

2.2.6 Statistical Analyses

Pearson's correlations and one-sample t-tests were performed using XLSTAT 2013 v1.01. $p \leq 0.05$ was considered statistically significant. Assay coefficient of variation (CV) was calculated as SD*100%/mean of T/S ratios. Intra-assay variability was derived from the CV between individual wells, and inter-assay variability was the CV calculated from the average value of duplicate measurements of LT-IC from multiple plates.

2.3 RESULTS

Our group transitioned from the monoplex qPCR to the MMqPCR assay for TL measurements after the publication of the latter. Although a strong correlation was initially obtained between MMqPCR and monoplex qPCR (Zanet *et al.*, 2013), we noticed over time an unacceptably high variability as well as several other issues that are described in detail below. This investigation was also partly prompted by changes in qPCR kits by manufacturers, and reaction mix variations between manufacturers.

2.3.1 $MgCl_2$ and EDTA Titration

We noticed the presence of non-specific peaks in the melting curve of MMqPCR reactions prepared with the described primers and thermal cycling profile, using the LightCycler® SYBRGreen I Master reaction mix as is, with no added $MgCl_2$ or EDTA (Figure 2.2A and 2.3A).

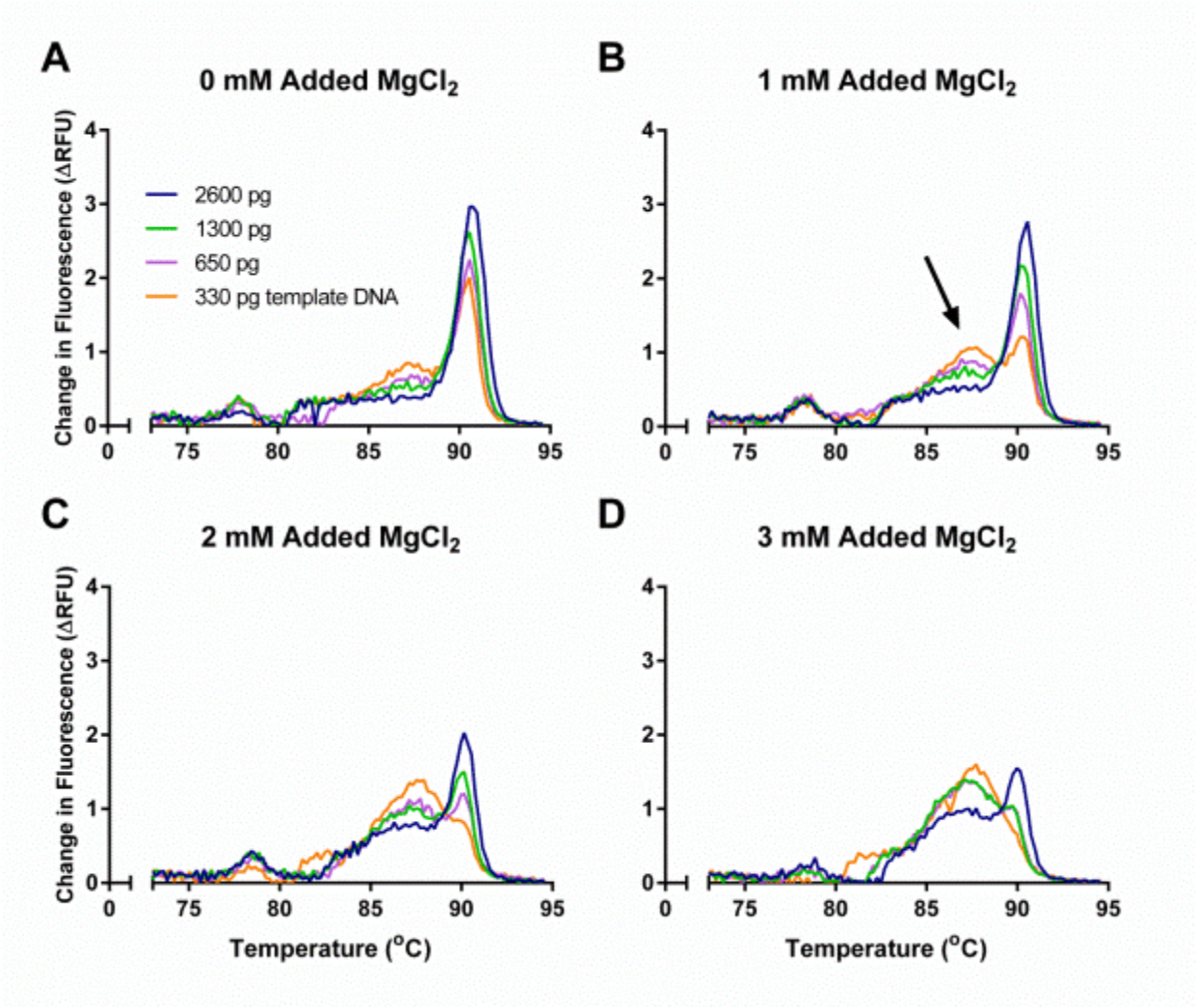

Figure 2.2. $MgCl_2$ titration melting curves. Melting curves were derived using change in relative fluorescence units (ΔRFU) per 0.2°C on various amounts of pooled human WB DNA. Melting curves for the following conditions are shown: (A) 0 mM, (B) 1 mM, (C) 2 mM, and (D) 3 mM added $MgCl_2$. The arrow indicates the dissociation peak of a non-specific peak present in all conditions.

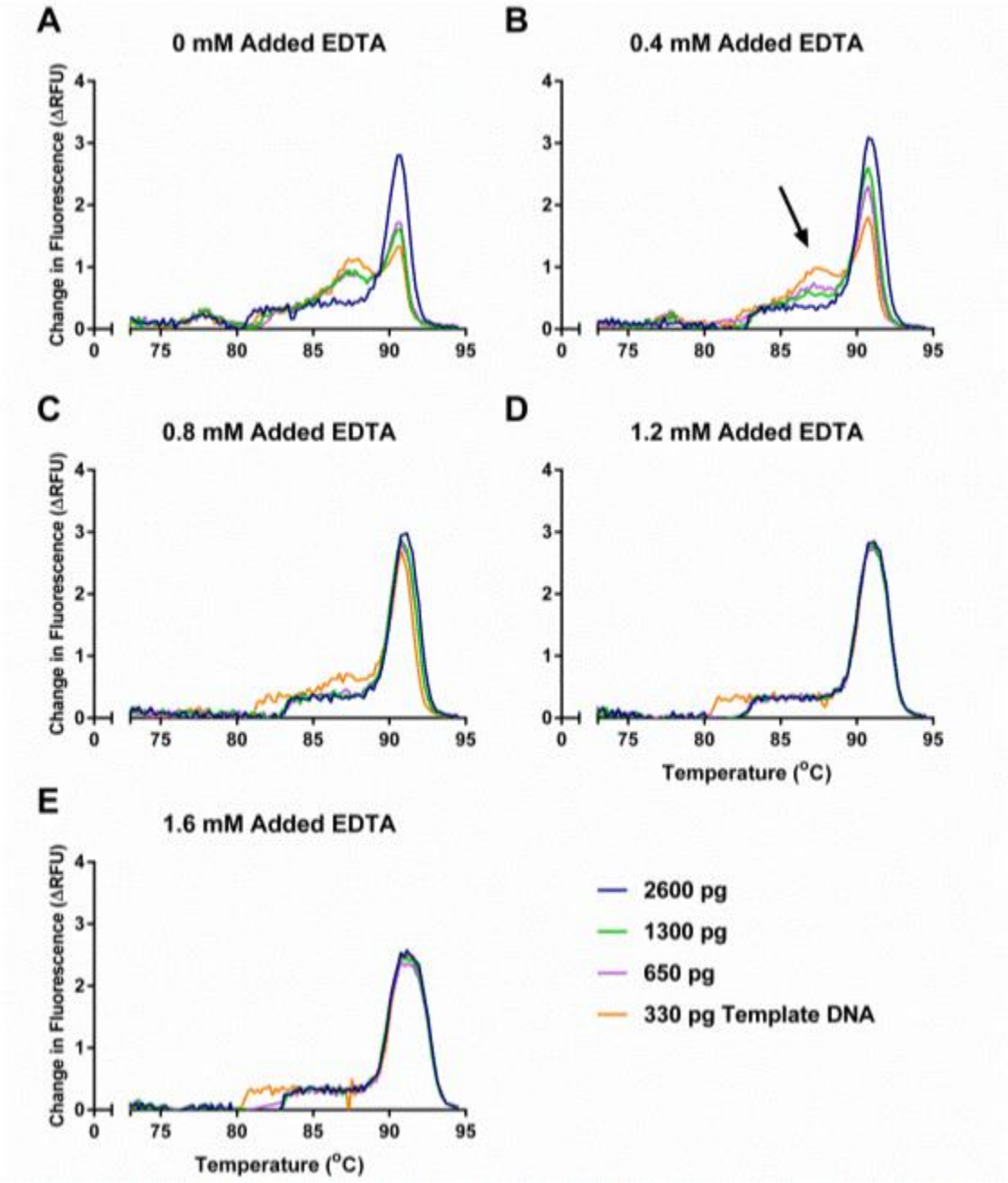

Figure 2.3. EDTA titration melting curves. Melting curves were derived using change in relative fluorescence units (ΔRFU) per 0.2ºC on various amounts of pooled human whole blood DNA. Melting curves for the following conditions are shown: (A) 0 mM, (B) 0.4 mM, (C) 0.8 mM, (D) 1.2 mM, (E) and 1.6 mM added EDTA. The arrow indicates the dissociation peak of a non-specific peak present in (A) through (C).

The intensity of these non-specific peaks was greater at lower DNA concentrations. This was accompanied by a PCR efficiency above 100% in the 88ºC acquisition (Figure 2.4A and 2.4B). The addition of $MgCl_2$ increased the prominence of the non-specific peaks (Figure 2.2 B-D) and further inflated the apparent 88ºC acquisition PCR efficiency (Figure 2.4A and 2.4B). Since the $MgCl_2$ concentration of the reaction mix was not disclosed by the manufacturer, an EDTA titration was performed to increase primer specificity by chelating free $MgCl_2$ present in the kit. The addition of EDTA decreased non-specific product peak (Figure 2.3) and normalized the 88ºC PCR efficiency to the desired range (Figure 2.4B). Furthermore, we found that $MgCl_2$ impeded amplicon peak fluorescence, whereas EDTA increased it (Figure 2.4C and 2.4D). The addition of EDTA to a final concentration of 1.2 mM gave optimal results for this specific kit.

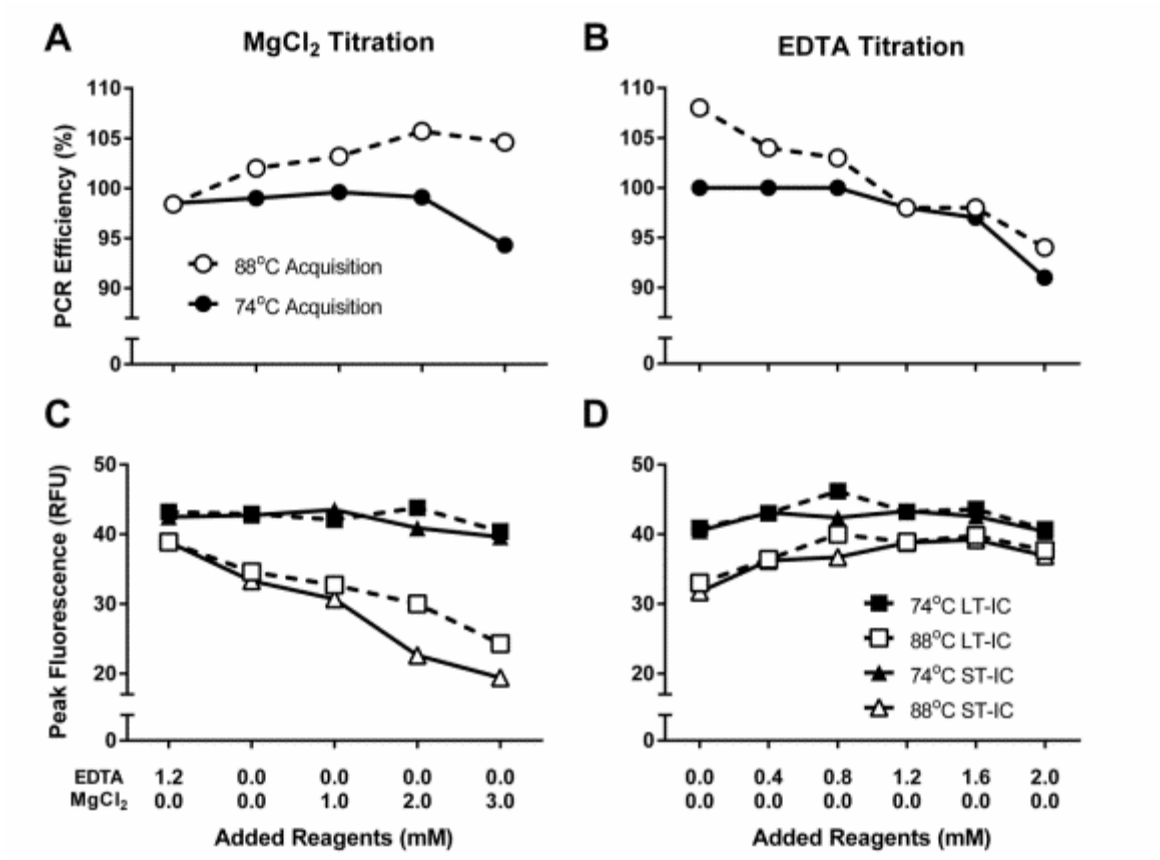

Figure 2.4. PCR efficiency and peak fluorescence for $MgCl_2$ and EDTA titrations. PCR efficiencies for both acquisitions were calculated using a single 8-point standard curve (R^2>0.99). PCR efficiencies for (A) $MgCl_2$ and (B) EDTA titrations are shown. Peak fluorescence was defined by the relative fluorescence unit (RFU) at the 40th cycle for both acquisitions and both ICs. Peak fluorescence values are shown for (C) $MgCl_2$ and (D) EDTA titrations. Each data point represents a single DNA sample.

2.3.2 Validating Amplicon Specificity

The specificity of the products was verified by gel electrophoresis (Figure 2.5) and melting curve analysis (Figure 2.6). In the agarose gel, amplicon band sizes reflect theoretical amplicon sizes, and are further verified through comparison with products obtained with individual telomere or albumin primer pairs (Figure 2.5). Primer dimers are visible only in the negative controls, with a C_T far higher (~33) than the established limit of quantification (~27.5). Reducing multiplex amplification to 30 cycles eliminates the band in the negative control while

preserving the desired products, indicating that primer dimers do not interfere with quantification of unknown DNA samples above the limit of quantification (Figure 2.5).

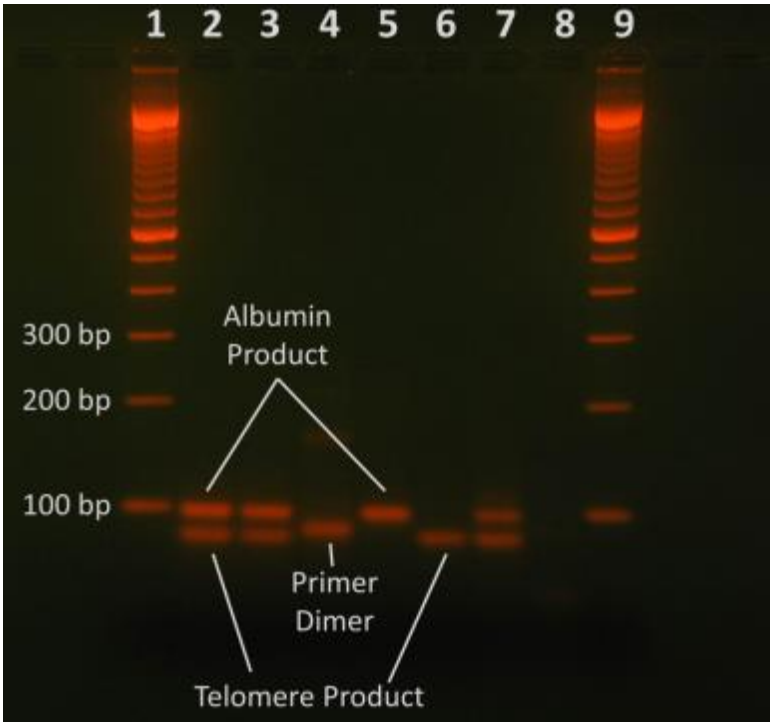

Figure 2.5. Visualization of amplicons on 3% agarose gel. Lanes 1 and 9 were loaded with 100 bp ladder. Lanes 2 through 6 are PCR products after the standard 40 cycle profile. Lane 2 is LT-IC with all primers, lane 3 is ST-IC with all primers, and lane 4 is negative control (using DNA elution buffer) with all primers. Lane 5 is LT-IC with only albumin primers, and lane 6 is LT-IC with only telomere primers. Lanes 7 and 8 are PCR products after only 30 cycles, with the rest of the program kept the same. Lane 7 is LC-IC with all primers, and lane 8 is negative control (DNA elution buffer) with all primers. All PCR products were diluted 1:10 prior to loading onto the agarose gel.

Melting curve analysis after the full 40 cycles yielded only a single peak representative of albumin amplicon dissociation. It is thus necessary to stop the program at 25 cycles to observe melting peaks of both telomere and albumin amplicons (Figure 2.6).

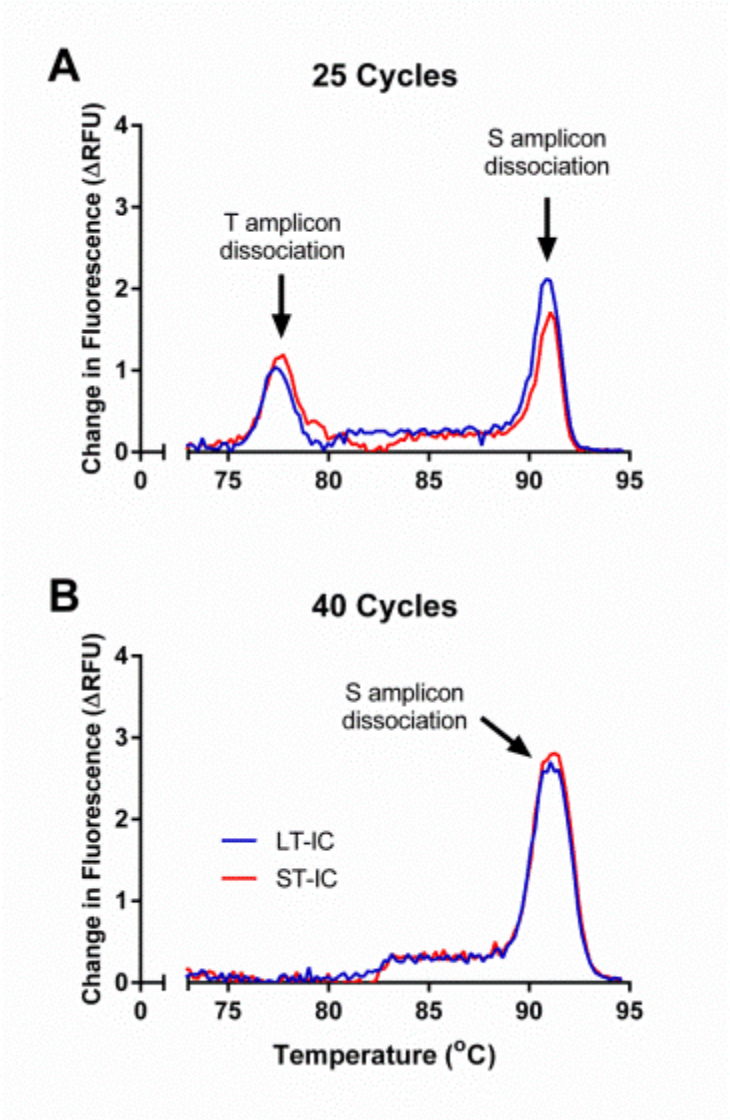

Figure 2.6. Telomere and albumin product melting curves after 25 and 40 cycles. Melting curves were derived using change in relative fluorescence units (ΔRFU) per 0.2ºC on LC-IC and ST-IC after (A) 25 and (B) 40 cycles.

2.3.3 Difference in C_T among Acquisitions

The T signal is derived from the 74°C acquisition that is composed of the additive fluorescence from both the telomere and the albumin amplicons present in the reaction at that stage. The assay relies on quantifying the T signal before the albumin signal rises above baseline. Thus, a sufficiently large T/S ratio in the sample (ensuring an adequate ΔC_T between the 74ºC

and 88ºC acquisitions) is essential to minimize the infringement of S in the T signal. In a DNA sample with critically short TL, the 88ºC acquisition is non-negligible when the 74ºC signal reaches C_T, thereby confounding the measured T/S (Figure 2.7).

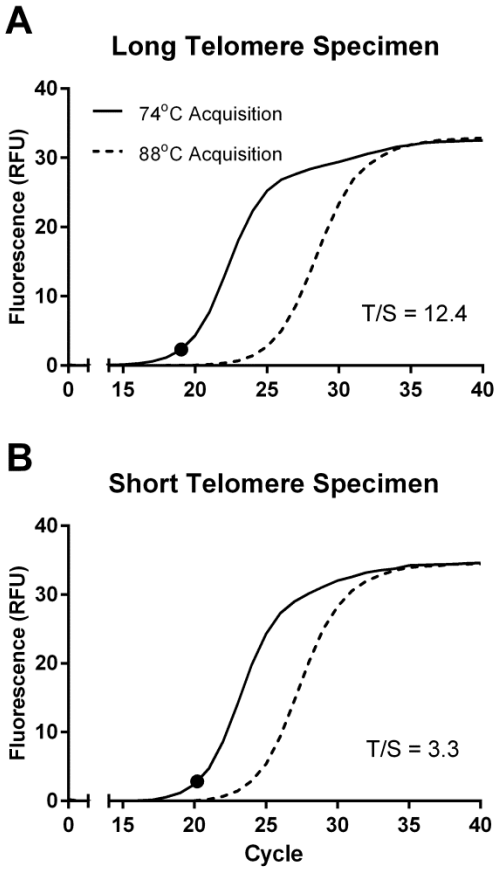

Figure 2.7. Fluorescence curves of long and short telomere specimens. Fluorescence curves of both acquisitions of a (A) long telomere placenta specimen and a (B) short telomere cord blood specimen are shown. The circles represent the C_T of the 74ºC acquisition.

To determine the minimum T/S that can be accurately measured with this protocol, we measured T/S ratio of the LT-IC and ST-IC using a series of standard curves built with varying T/S ratios. The standard curves were created with DNA extracted from placenta, which has naturally long telomeres, spiked with varying amounts of a plasmid containing the albumin gene.

It was found that LT-IC T/S ratios calculated based on standard curves with low T/S ratios are underestimated (Figure 2.8A). The sizable amount of albumin amplicon fluorescence in the 74ºC acquisitions of a low T/S standard curve could not serve as an accurate reference for the relatively high T/S LT-IC, in which the presence of albumin amplicon fluorescence in the 74ºC acquisitions is negligible. Figure 2.8A shows that LT-IC T/S ratio stabilized when the standard curve's T/S ratio was approximately >6, indicating that albumin fluorescence signal in the 74ºC acquisition became negligible beyond this point, and that a sufficient ΔC_T had been reached. These results were reproduced using ST-IC (data not shown) revealing the same minimum T/S threshold. Therefore, this minimum T/S ratio must be present in the standard curve and in all samples to ensure a valid output with this assay.

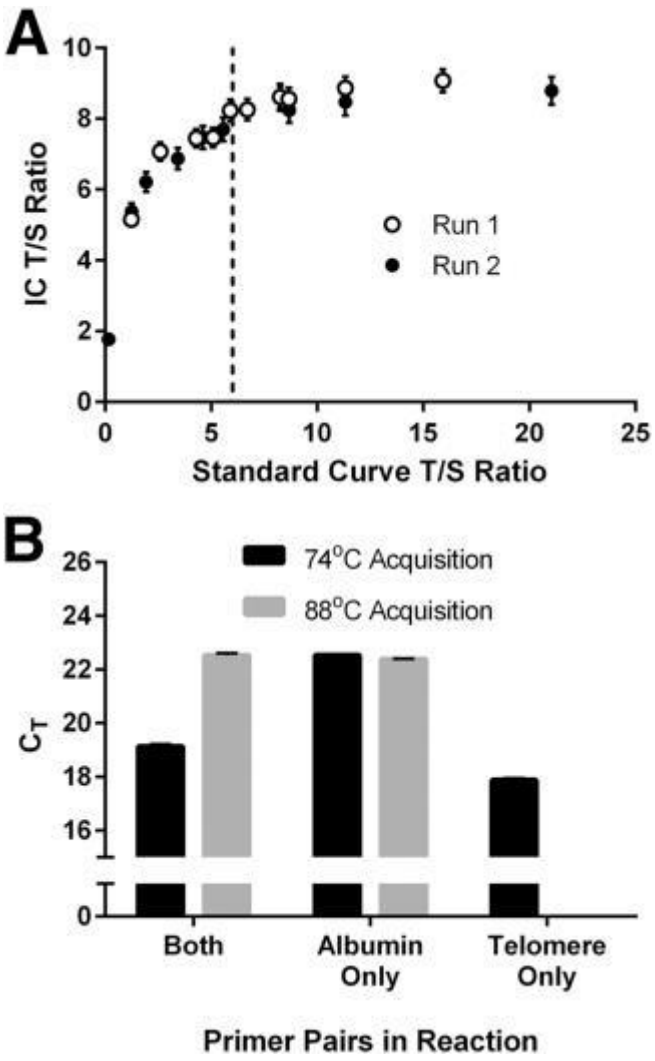

Figure 2.8. Minimum quantifiable telomere/single-copy nuclear gene (T/S) ratio and amplification with individual primer pairs. **A:** T/S ratios of long telomere internal control (LT-IC) were calculated using standard curves prepared from placenta DNA, a tissue with LTs, spiked with varying quantities of a plasmid containing the *ALB* amplicon (without GC clamps). Theoretical standard curve T/S ratios are shown on the *x* axis and IC T/S ratios on the *y* axis. IC T/S ratios were from two runs, each containing six technical replicates. The vertical dotted line marks the minimum quantifiable T/S ratio, which is 6. **B:** Threshold cycle (C_T) values of both acquisitions are shown when the reaction contained the following primer combinations: albu+albd+telg+telc, albu+albd only and telg+telc only. Data points are derived from six to eight technical replicates. Error bars indicate SDs.

To directly quantify the albumin fluorescence signal in the 74°C acquisition, albumin primers were removed from the MMqPCR reaction (Figure 2.8B). This resulted in a lower telomere C_T, revealing that telomere amplification may be partially inhibited by the presence of

albumin primers. Consequently, albumin amplicon fluorescence in the 74°C acquisition could not be directly quantified. The assay was also performed in the absence of telomere primers from the reaction to verify that telomere amplicons are completely dissociated prior to the 88°C acquisition (Figure 2.8B). The C_T of albumin was unchanged, indicating that no telomere amplicon is present during the 88°C acquisition.

2.3.4 Variability and Limit of Quantification

Although the linear range of this assay extends past the lowest point on the standard curve, increased variability was observed below this point (Figure 2.9). This is likely due to stochastic sampling effects such that sampling becomes governed by Poisson distributions. In addition, rejection due to QC failure is high at these low DNA concentrations. This effect is driven by variability in the amplification of albumin, which is present at lower abundance. To avoid these sampling errors, the limit of quantification for our assay was thus defined at the concentration of the last standard: 160 pg/μl. This is equivalent to a C_T of 27.5 in the 88°C acquisition. As such, the defined standard curve serves a dual purpose by ensuring that data are being collected both in the linear range of this assay for each gene and above the limit of reliable quantification. Of note, since C_T determination does not take into account the first two cycles of amplification at 49°C, the 27.5 C_T underestimates the true number of doublings that have occurred in the entire reaction.

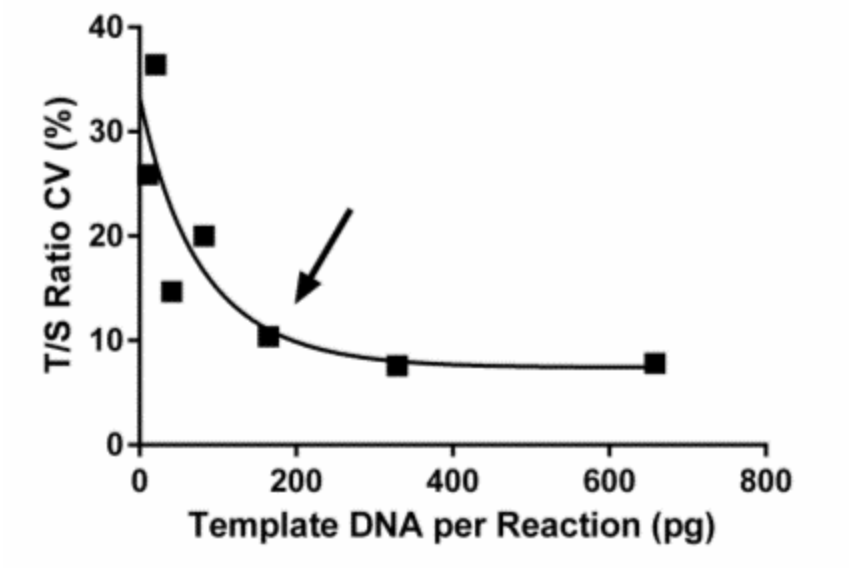

Figure 2.9. Limit of quantification. The T/S ratios of various amounts of pooled human whole blood DNA were measured. Each data point represents the CV calculated from the T/S ratio of 10 technical replicates. The arrow indicates the concentration of the limit of quantification: Approximately 160 pg of DNA per reaction.

We calculated the average percent difference between T/S ratio duplicates for all samples in each run, and used this data to detect any random or transient plate-wide sources of variability. If this average exceeds 10%, the run is rejected if it violated any of the other four QC criteria. In our experience, this resulted in a 7% rejection rate (7 out of 100 runs). Similar transient and unsystematic errors that might only affect individual random wells were addressed by rejecting samples with T/S ratios that differed by >15% between duplicates. In two of our recent studies (both with n≥1000) with two different operators, these errors resulted in the initial rejection of approximately 4% of all samples. However, as per our QC protocol, these samples were repeated in duplicate, and ultimately all yielded usable data.

The fluctuation and linearity of T/S across a range of DNA concentrations was investigated using the standard curve, as this could represent another source of variability. To monitor this and determine the range over which the T/S ratio is stable, both the standard curves

and the T/S *vs*. S linear regression were plotted for each run. Standard curves that appear seemingly linear (R^2>0.99) can nevertheless exhibit T/S ratios that fluctuate with varying DNA concentrations (Figure 2.10). This is presumably due to dissimilarities in PCR efficiencies for the two amplicons. Based on a large number of runs, it was determined that as part of QC criteria, the difference between the two PCR efficiencies should not exceed 2.5% or 0.05-fold amplification per cycle. In our experience, the mean T/S ratio CV across the 8 standards included in each run was approximately 7% after 64 runs by three different operators.

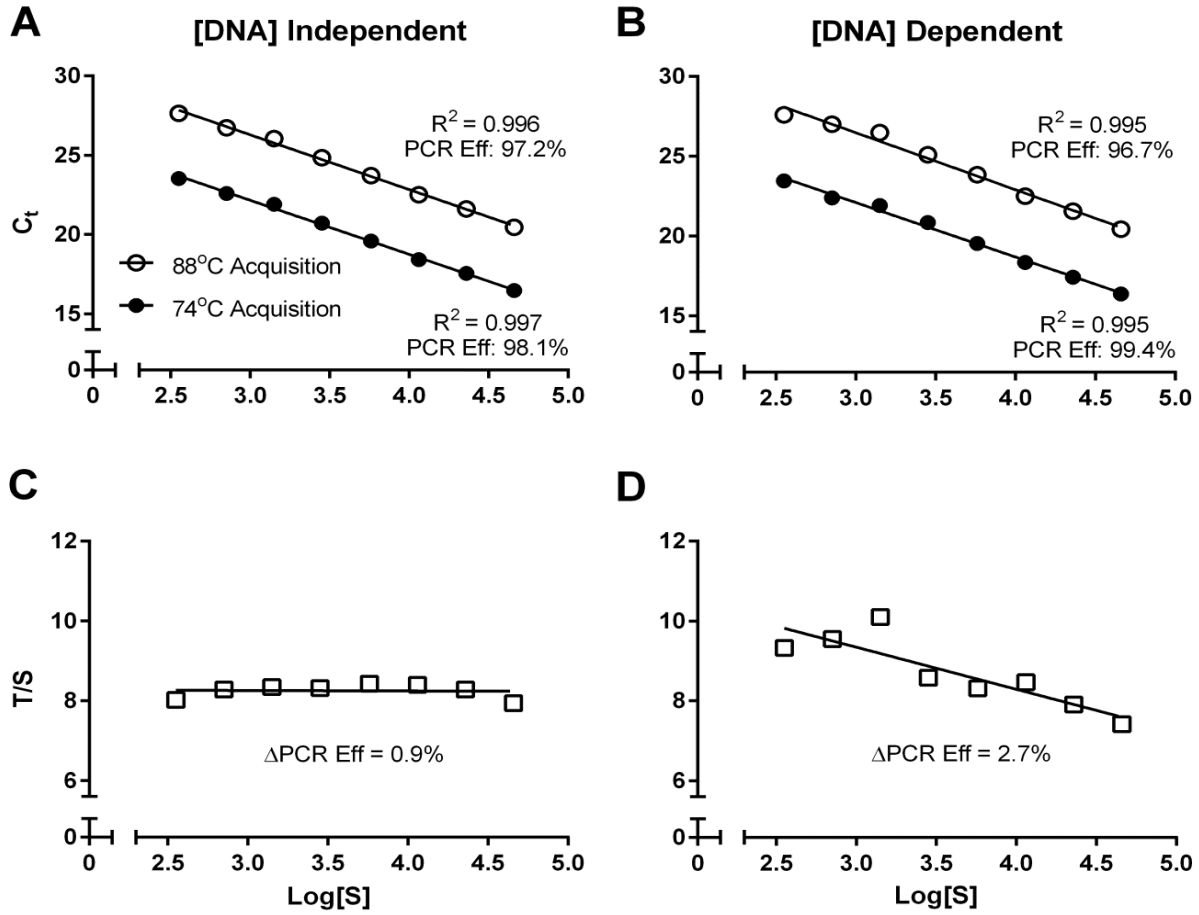

Figure 2.10. Impact of small fluctuations in standard curves. Both acquisitions of two very linear standard curves (A) and (B) are shown as examples. Log [S] on the x axis is derived from the logarithm of albumin copy numbers of each standard. Pearson's R^2 and PCR efficiencies are shown. (C) T/S ratios from the DNA independent standard curve in (A) are shown. (D) Small fluctuations in the standard curve of (B) drive DNA dependence in T/S ratio. Differences in PCR efficiency between the two acquisitions are shown.

2.3.5 Reproducibility of the MMqPCR Assay

To examine the intra-assay and inter-assay reproducibility of T/S measurements by MMqPCR, T/S ratios of the LT-IC were used. LT-IC assayed in 91 wells of a single run was used to establish an intra-assay CV of 6.2%. In a separate run with a different operator, and using just 14 wells avoiding the edge of the plate, an intra-assay CV of 4.2% was observed. To examine inter-assay reproducibility, we determined the CV of LT-IC T/S ratios of three different studies by three different operators over a period of 2 years. Inter-assay CVs were measured at 3.2% (n=27 runs), 4.3% (n=38 runs), and 4.9% (n=9 runs). Plate position effects were quantified by column, row, and individual wells (Figure 2.11).

A

Row \ Column	1	2	3	4	5	6	7	8	9	10	11	12	Average
A	13	12	10	14	6	7	3	3	8	6	5	5	8
B	21	8	3	7	4	2	5	4	2	1	3	3	5
C	14	7	2	2	2	4	5	6	3	2	3	3	4
D	10	5	4	4	5	4	-	9	9	4	2	1	5
E	9	4	3	8	8	3	7	13	9	4	8	1	7
F	4	7	3	6	4	8	8	8	9	5	6	4	6
G	5	8	3	4	3	6	9	4	3	3	2	3	5
H	7	2	4	2	5	4	4	1	5	7	4	2	4
Average	10	7	4	6	5	5	6	6	6	4	4	3	

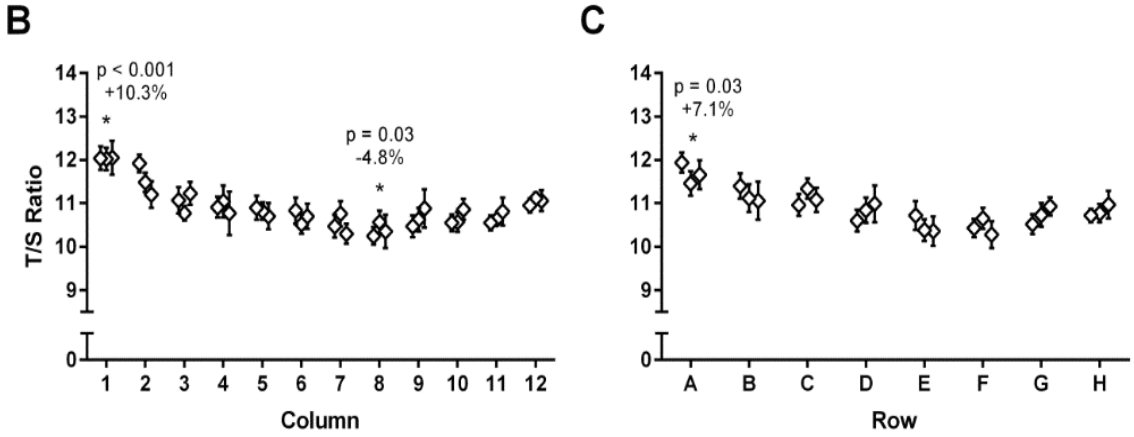

Figure 2.11. Plate position effects. LT-IC was measured in every well on 96-well plates, except well D7, which was used as a negative template control. (A) The absolute difference in CV (%) between the T/S ratio of each well and the plate average is shown. This represents a roughly normal distribution with approximately half of the wells deviating above the plate average. The average of the data from each column and row are shown. Variability across (B) columns and (C) rows are shown. Error bars represent standard deviation calculated from the number of replicates in each column (8) or each row (12). Columns or rows significantly different from plate average (using one-sample T-test) are marked, with p values and effect sizes shown. Average effect sizes are displayed as the % difference of each row or column compared to the average of the plate. These data are derived from the average of three runs.

2.3.6 Concordance between Relative TL Measured Using MMqPCR *vs.* Other Methods

2.3.6.1 Concordance between Relative TL Measured Using MMqPCR *vs.* Flow-FISH

Flow-FISH was performed on the lymphocyte and granulocyte subsets in cord blood specimens (n=33) covering a 2-fold range of T/S ratios. A strong correlation was observed between both lymphocyte and granulocyte TL measured by Flow-FISH and LTL by MMqPCR (R^2= 0.81 and 0.71, p< 0.0001) (Figure 2.12A). We previously collected LTL data on a subset of these cord blood specimens (n=24) using monoplex qPCR. Similar correlations were observed between lymphocyte and granulocyte TL measured by monoplex qPCR and Flow-FISH (R^2= 0.82 and 0.70, p< 0.0001) (Figure 2.12B).

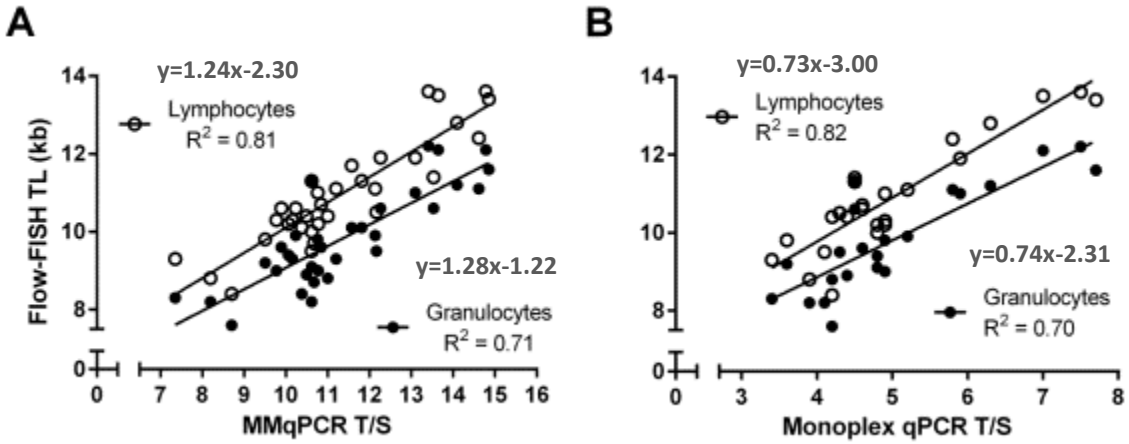

Figure 2.12. Pearson's correlation between Flow-FISH and MMqPCR.TL in cord blood samples (n=33) were measured by Flow-FISH, MMqPCR, and monoplex qPCR. Depicted are the (A) correlations between mean MMqPCR LTL and median Flow-FISH lymphocyte and granulocyte TL, as well as (B) correlations between monoplex qPCR LTL and Flow-FISH lymphocyte and granulocyte TL. Pearson's R^2 is shown, all p values are <0.0001.

Correlations show that the measurements by two methods are significantly related but this would be expected given that the measures should be highly related. The slopes and intercepts of the linear regression equations on Figure 2.12 indicate a difference between the measurements. This difference (or bias) was expected since two methods were not on the same scale. Bland and Altman (B&A) plots (Bland & Altman, 1986; Giavarina, 2015) were used for illustrating disagreement between Flow-FISH and MMqPCR mthods (Figure 2.13).

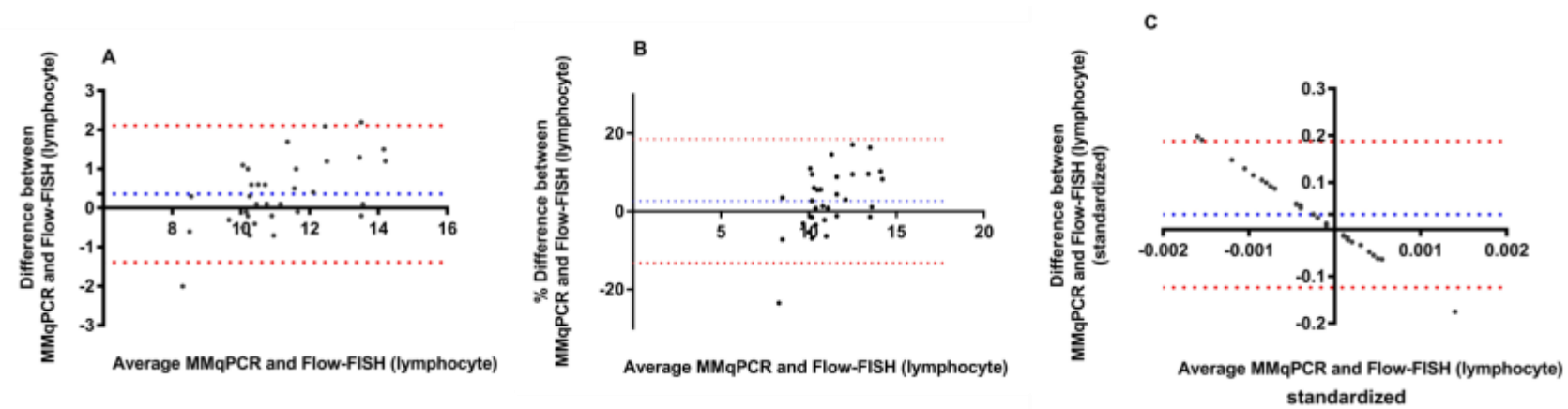

Figure 2.13. Bland and Altman (B&A) plots for comparison between Flow-FISH and MMqPCR.TL in cord blood samples (n=33) (lymphocyte) were measured by Flow-FISH, MMqPCR. The B&A plots were done on difference between Telomere measures (A); the % difference between the Telomere measures (B); and the difference between standardized Telomere measures, according to [(X-$\bar{X}$)/SD] (C). Blue dotted lines correspond to mean of differences between two methods and red dotted lines represent the limits of agreement as defined by the mean ± 1.96 SD.

Figure 2.13 A, shows the difference between the T/S ratio measurements by MMqPCR and Flow-FISH against the average of these measurements. In an ideal condition, the differences between two methods should be equal to zero. However, any method implies some degree of error. Our B&A plots show that a bias exists whereby MMqPCR values are T/S ratios and Flow-FISH values are TL in kb. Approximately 91% of the data points lie within the mean ± 1.96 SD, which is close to the general rule stipulating that 95% of data should do so (Bland & Altman, 1986; Giavarina, 2015). However, our data suggest that the difference between these methods is proportional to the magnitude of values. This is not unexpected given that we know that variability (hence disagreement) increases as TL values increase. If we express the differences as percentages of the values against the magnitude of measurements, as presented in Figure 2.13 B, the bias remains, and 94% of the data points are between the aforementioned lines of agreement. In Figure 2.13 C, the TL values from both methods were standardized in order to represent them on a comparable scale. The differences between standardized values for both methods were then plotted against the average of the standardized values (units are now SD). This eliminated the systematic bias induced by the different scales of the assays.

2.3.6.2 Concordance between Relative TL Measured Using MMqPCR *vs.* TRF

TRF was used to determine the absolute TL (in kb) in placenta specimens (n=21) and cultured HT-29 cells treated with HIV ARVs (n=36). Placenta is a tissue with long TL, and the HT-29 cells have artificially reduced TL following exposure to the ARVs. Together, they represent a 4-fold range of T/S. A strong correlation was observed between relative TL measured by MMqPCR and the absolute TRF values (R^2= 0.88, p< 0.0001) (Figure 2.13A), and a similar correlation was seen between the monoplex qPCR and TRF values (R^2= 0.85, p< 0.0001) (Figure 2.13B).

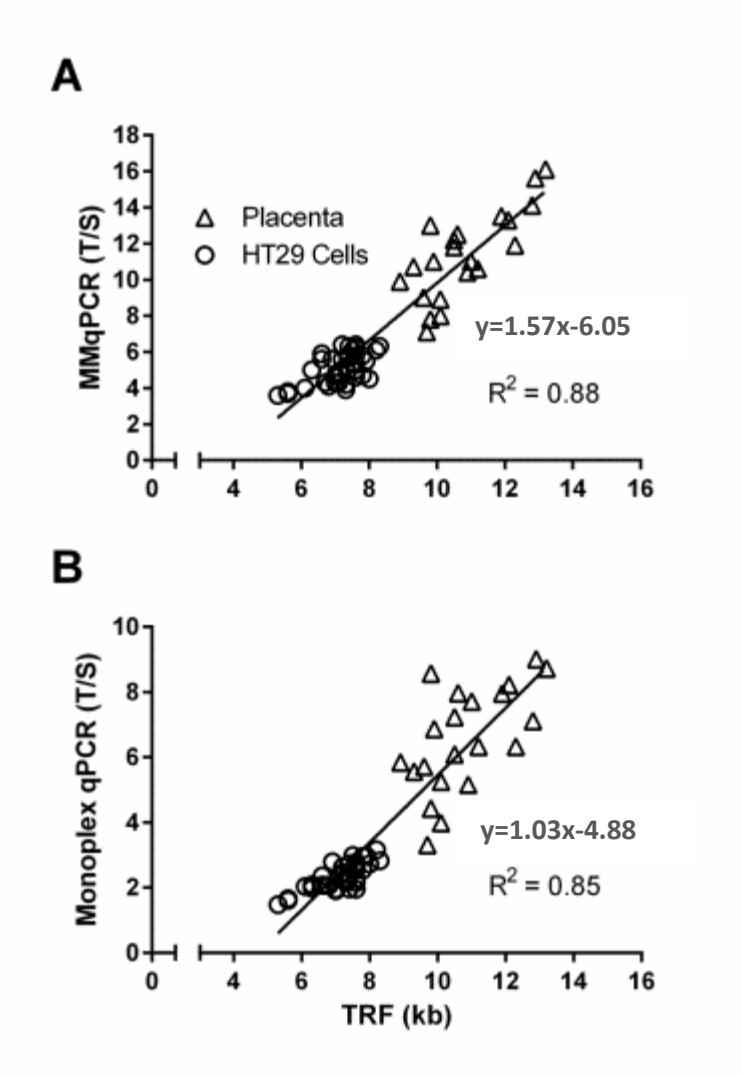

Figure 2.14. Pearson's Correlation between TRF and qPCR.TL in placenta (n=21) and HT-29 cell culture (n=36) were measured by TRF Southern blot and qPCR. TRF data were compared with (A) MMqPCR and (B) monoplex qPCR. Pearson's R^2 is shown, all p values <0.0001.

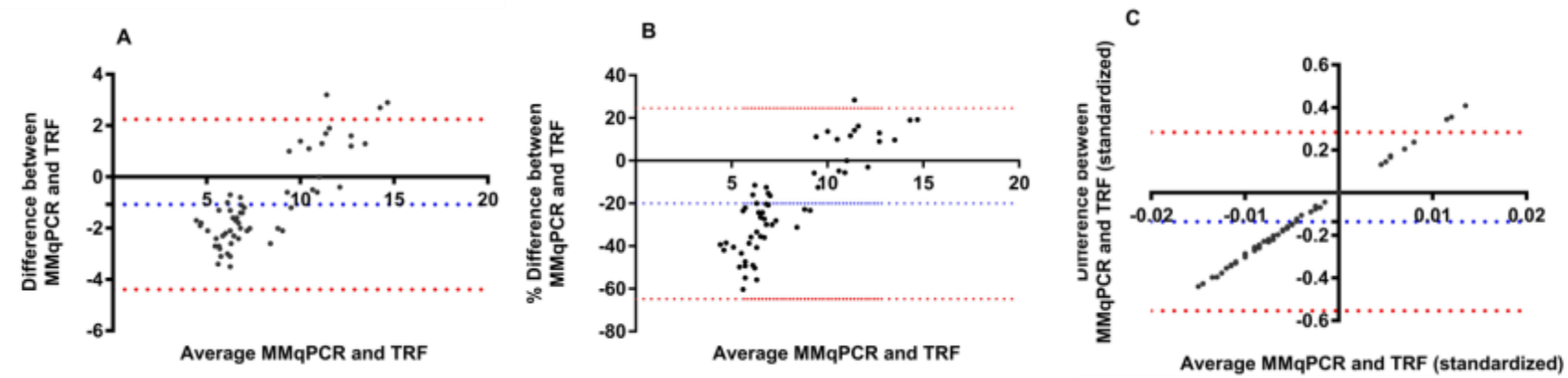

Figure 2.15. Bland and Altman (B&A) plots for comparison between MMqPCR and TRF. TL in placenta (n=21) and HT-29 cell culture (n=36) were measured by TRF Southern blot and MMqPCR. The B&A plots were done on difference between TL measures (A); the % difference between the TL measures (B); and the difference between standardized TL measures, according to [(X-$\bar{X}$)/SD] (C). Blue dotted lines correspond to mean of differences between two methods and red dotted lines represent the limits of agreement as defined by the mean ± 1.96 SD.

The measurements by MMqPCR and TRF are significantly correlated. However, B&A plots show that a bias also exists between MMqPCR and TRF methods, but approximately 95% of the data points lie within the mean ± 1.96 SD, which is very similar to the general rule stipulating that 95% of data should do so (Bland & Altman, 1986; Giavarina, 2015). In Figure 2.15 B, the bias remains, and 98% of the data points are between the lines of agreement.

2.3.6.3 Concordance between Relative TL Measured Using MMqPCR *vs.* Monoplex qPCR

Monoplex and MMqPCR were performed on DBS (n=58) and cord blood (n=26) spanning a 3-fold range of T/S ratios. A strong correlation was observed for each individual gene (S, R^2= 0.78, p<0.0001; T, R^2= 0.66, p< 0.0001), as well as between the T/S ratios (R^2= 0.67, p< 0.0001) as measured by the two qPCR methods (Figure 2.14). Similar correlations were observed in each subset of DBS and cord blood samples (Figure 2.16).

The measurements by two assays are significantly correlated. The slopes and intercepts of the linear regression equations on Figure 2.16 indicate a difference between the measurements. This difference (or bias) was expected since two methods were not on the same scale

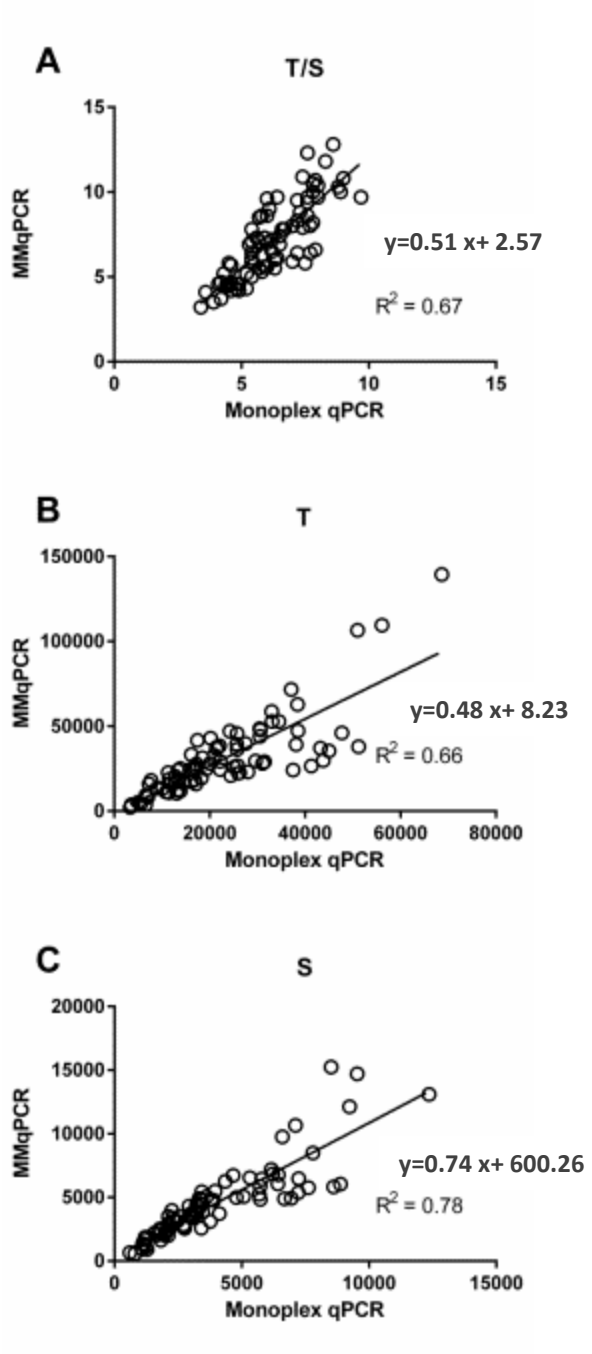

Figure 2.16. Pearson's Correlation between monoplex qPCR and MMqPCR.TL in dried blood spot samples (n=58) and cord blood samples (n=26) were measured by monoplex qPCR and MMqPCR. Correlations between the two techniques were done on (A) T/S, (B) T, and (C) S, using both sample sets combined (n=84). Pearson's R^2 is shown, all p values <0.0001.

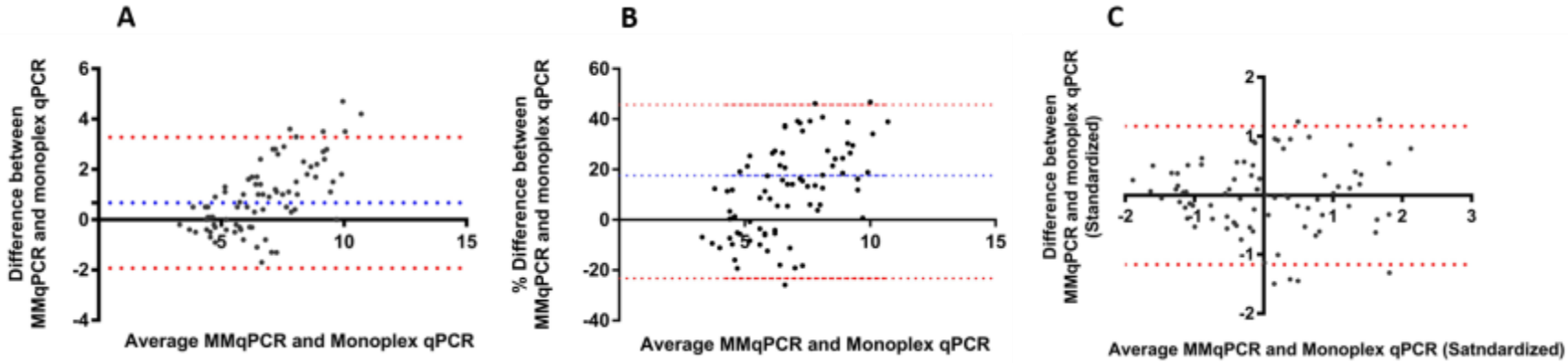

Figure 2.17. Bland and Altman (B&A) plots for comparison between monoplex qPCR and MMqPCR. TL in dried blood spot samples (n=58) and cord blood samples (n=26) were measured by monoplex qPCR and MMqPCR. The B&A plots were done on difference between relative TL measures (T/S ratios) (A); the % difference between the T/S ratios (B); and the difference between standardized T/S ratios, according to [(X-$\bar{X}$)/SD] (C). Blue dotted lines correspond to mean of differences between two methods and red dotted lines represent the limits of agreement as defined by the mean ± 1.96 SD.

The B&A plots show that a bias exists whereby MMqPCR values are systematically higher than monoplex values, but also that 93% of the data points lie within the mean ± 1.96 SD, which is close to the general rule stipulating that 95% of data should do so (Bland & Altman, 1986; Giavarina, 2015). However, our data suggest that the difference between methods is proportional to the magnitude of values. This is not unexpected given that we know that variability increases as TL values increase.

In Figure 2.18 B, the bias remains, and 96% of the data points are between the aforementioned lines of agreement. In Figure 2.18 C, the TL values from both methods were standardized in order to represent them on a comparable scale. This eliminated the systematic bias induced by the different scales of the assays. Overall, B&A plots did not suggest that one technique is superior to the other one, but suggest that differences between these techniques are between commonly applied lines of agreements.

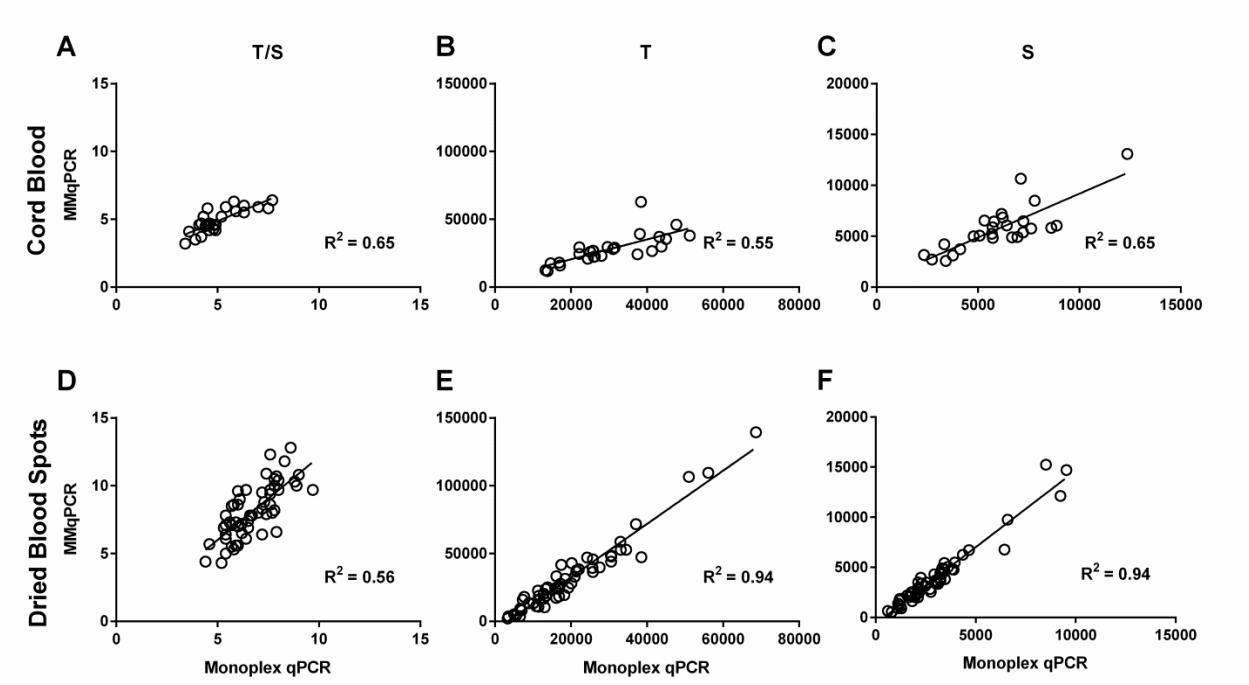

Figure 2.18. Pearson's correlation between monoplex qPCR and MMqPCR. TL in cord blood (n=26) and dried blood spot (n=58) samples were measured by monoplex and MMqPCR. Correlations between the two techniques were done on (A) T/S, (B) T, (C) and S for cord blood; and (D) T/S, (E) T, (F) and S for dried blood spots. Pearson's R^2 is shown, all p values <0.0001

2.4 Discussion

Although qPCR-based TL assays offer many substantial advantages for high-throughput studies, commercial applications, and potentially routine health diagnostics, they have often been described as less precise than other established methods such as Southern blot, Flow-FISH, Q-FISH, and Single Telomere Length Analysis (STELA) (Aviv *et al.*, 2011; Aubert *et al.*, 2012; Martin-Ruiz *et al.*, 2015). As such, there is a demand in the literature for increased robustness, as well as more rigorous quality control and calibration when using TL assays based on qPCR (Jodczyk *et al.*, 2015; Simon *et al.*, 2015). Therefore, improvements to reduce variability and increase reproducibility were a leading priority during our optimization of this assay.

The primary impetus behind this report was to share our experience in implementing this assay and perhaps aid others in the adoption of the MMqPCR method to measure TL. A substantial contribution towards this goal is the use of a purchased one-component reaction mix which decreases the number of reagents while facilitating high-throughput implementation. Purchased reaction mixes are subject to industry-standard quality control, which contributes to lowering inter-assay variability, especially within a given lot. They may also decrease inter-operator variability owing to the lower complexity of the reaction mix preparation. In contrast, the publication of the original MMqPCR technique describes a method involving eight separate reagents (Cawthon, 2009), excluding primers and sample DNA. Six years later, numerous studies continue to use convoluted multi-reagent versions of MMqPCR. Few studies describe the use of commercial kits with MMqPCR, and those that do may not include the level of methodological detail that would facilitate a wider adoption of the assay (Buxton *et al.*, 2011; Seguí *et al.*, 2013; Srinivasa *et al.*, 2014; Zhu *et al.*, 2015). Here, we provide detailed methodology for a kit-based MMqPCR TL assay on the LightCycler 480, using the same primers as those originally described, in a reaction mix that involves only two components apart from primers and template DNA.

In our experience, it was necessary to add EDTA to the Roche kit to bolster primer specificity and eliminate non-specific products. Conversely, the addition of surplus $MgCl_2$ worsened non-specific peaks (Figure 2.2) in the melting curve. These effects were not initially expected as manufacturers often recommend that users determine experimentally how much $MgCl_2$, rather than a chelator such as EDTA, should be added to their reaction. As this effect is likely kit-dependent, melting curve titration analyses may help determine each kit's optimal divalent cation concentration.

Adhering to certain criteria with respect to DNA concentration was found to reduce assay variability and improve reproducibility. For example, one such restriction is the lower limit of DNA concentration, below which a substantial increase in variability is observed due to stochastic sampling error. This may be resolved by increasing the number of technical replicates and employing more rigorous QC (Concetti *et al.*, 2015), although variability seems to rise rapidly with decreasing DNA concentration. Others have reported required DNA sample quantity for MMqPCR to be in the range of nanograms (Aubert *et al.*, 2012; Montpetit *et al.*, 2014), and studies within the past few years describe protocols performed on 5-40 ng of cellular DNA (Buxton *et al.*, 2011; Lan *et al.*, 2013; Weischer *et al.*, 2014; Blackburn *et al.*, 2015; Ramunas *et al.*, 2015). Here, we report the lower limit of DNA for robust quantification by MMqPCR to be several folds lower, at 160 pg of cellular DNA. This may enable TL studies on very small specimens such as blood droplets or micro-dissection material.

Based on our data, it is clear that assaying samples with very low average TL will result in an overestimated T/S ratio because of albumin signal present in the 74ºC acquisition. Under our assay conditions, this occurs when T/S ratios are approximately ≥6, as defined by our standards. Due to the lack of universal ICs or standard curves, measured T/S ratios are not directly comparable between labs and as such, it may be beneficial for others wishing to adopt this assay to set a minimum acceptable ΔC_T between the 74ºC and 88ºC acquisitions. Here, the lower limit in ΔC_T between the two acquisitions was approximately 3.6. Optimally, we suggest that users wishing to adopt this assay conduct a standard curve T/S ratio titration to determine the lower T/S ratio limit under which the assay loses robustness.

Efforts to subtract the undesired albumin signal from that of the 74ºC acquisition signal in silico to obtain a pure telomere signal have yielded data with an equivalent or weaker

relationship to reference monoplex and Flow-FISH assays (data not shown). However, to measure DNA samples with extremely low average TL, it may be possible to adjust for the logarithmic pattern reported here (Figure 2.7A), in order to arithmetically remove the systematic bias caused by albumin signal in the 74°C acquisition. Otherwise, it may be necessary to revert to the monoplex method for measuring samples with very short average telomeres. Previous efforts in our lab to optimize the MMqPCR using unbalanced primer concentrations (3:1 ratio of telomere to albumin primer) yielded larger ΔC_T between acquisitions. However, we found those conditions subject to higher variability and lower correlation with Flow-FISH compared to balanced primer concentrations (data not shown). Nevertheless, such concessions could be made for the purpose of measuring average telomere lengths that are shorter than the assay's T/S ratio limit of stability using MMqPCR.

In our experience, the LightCycler® 480 instrument produces fast, sensitive, and reproducible measurements, with minimal edge effects when used for MMqPCR. Others have also reported on its comparative speed and sensitivity (Deback *et al.*, 2009; Przybylski *et al.*, 2012). However, it has the distinct disadvantage that to date, its software is incapable of performing analyses on multiple acquisitions per cycle, as needed for MMqPCR. This therefore requires the use of external software such as LinRegPCR to process data, adding a convoluted step to the procedure and introducing a potential source of operator error. Although this LightCycler disadvantage can be mitigated by tools such as a Microsoft Excel macro, other qPCR instruments typically include software with the ability to delineate groups of acquisitions within a cycle, allowing MMqPCR data processing with software built into the instrument, significantly reducing procedural complexity and operator workload.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) published in 2009 is a useful and comprehensive reference for qPCR considerations (Bustin *et al.*, 2009). However, the nature of MMqPCR necessitates considerations beyond those relevant to conventional qPCR. A key example is the determination of the lower T/S ratio limit mentioned earlier. Another potential concern is dissimilarity between efficiencies of the 74ºC and 88ºC acquisitions. Uneven efficiencies between T and S can introduce variability in T/S ratios, especially near the extremes of the expected linear range of quantification. Apart from determining PCR efficiencies, the standard curve can be used to monitor the T/S ratio's dependence on DNA concentration. Despite highly linear regression of individual acquisitions of the standard curves, the actual T/S ratio may still deviate between standards (Figure 2.10). Such an effect may be missed if ICs have T and S measures that fall in the middle of the standard curves. Therefore, in addition to recording the difference in PCR efficiencies between the two acquisitions, it may be necessary to incorporate more than one IC to cover the range where this effect might be more prominent. We aimed for a T/S ratio CV of less than 10% across the 8 standards of the standard curve included in each run. If this variability increases over time, it may indicate inadequate storage conditions of the standards. Other potential causes for further deviation include extension past linear range, amplification of non-specific products, or as mentioned above, non-collinear 74ºC and 88ºC standard curves.

As in all intercalator dye-based qPCR assays, the specificity of the primers for the desired MMqPCR amplicons is crucial and was verified here through agarose gel electrophoresis. As shown in the original publication, shortening the cycling program from 40 to 25 cycles is necessary to visualize the dissociation of both amplicons on the melting curve (Cawthon, 2009). Indeed, in melting curve analyses performed after the full 40 cycle program (Figure 2.6B), only

the albumin product dissociation is visible. It is presumed that after 40 cycles, the two amplicons become sufficiently abundant to saturate the dye. Thus, when temperature rises above T product dissociation, released SYBR green binds to free double stranded albumin amplicon, thereby stifling the T amplicon's dissociation peak in the melting curve.

Comparison of TL measurements across different techniques is required to validate the MMqPCR. The original publication previously showed a strong correlation (R^2= 0.84) between relative TL in WB DNA samples (n=95) measured by MMqPCR and the mean TRF lengths by the traditional Southern blot approach (Cawthon, 2009). This correlation is higher than the correlation for relative TL in the same samples determined by monoplex qPCR *vs*. their mean TRF lengths (R^2= 0.68) (Cawthon, 2009). In our study, we found similarly strong correlations comparing TRF against both qPCR techniques (R^2= 0.88 and R^2= 0.85) when considering DNA samples with a wide range of TL. Robust correlations were also seen for both monoplex and MMqPCR *vs*. Flow-Fish data in cord blood, irrespective of the subset of the cells (lymphocytes *vs*. granulocytes, R^2 = 0.70-0.82). Our data suggest that concordance between MMqPCR and alternative techniques is not greatly improved compared to monoplex qPCR, when examined using a several tissues with varying TL. However, MMqPCR is still considerably more time- and labour-efficient, at approximately half the cost.

We previously reported a strong correlation for relative TL (n=32) determined by monoplex and MMqPCR assays (R^2= 0.65) (Zanet *et al.*, 2013). Here, MMqPCR and monoplex qPCR assays across a wide range of samples (n=84) were compared once again and showed similar concordance (R^2= 0.67).

The range of intra-assay and inter-assay variability reported for qPCR has been broad compared to other techniques (Zanet *et al.*, 2013; Martin-Ruiz *et al.*, 2015), and its reporting in

the literature is inconsistent. However, a previous study reported no statistically significant difference in intra-assay or inter-assay variability between qPCR, Southern Blot, or STELA in aggregate data across 10 labs (Martin-Ruiz *et al.*, 2015). It further showed that while the absolute relative TL values from different laboratories differed widely, their ranking were highly correlated (correlation coefficients of 0.63–0.99) (Martin-Ruiz *et al.*, 2015). We suggest that better understanding sources of variability and addressing them with appropriate optimization and QC should lower MMqPCR variability to the range reported for other techniques. We also strongly believe that intra-assay and inter-assay variability should be included in all publications involving MMqPCR TL assays.

2.5 Strengths and Limitations

Although MMqPCR is quickly becoming the most attractive method for TL measurements, it is limited by the fact that it only measures average TL. Unlike methods such as Flow-FISH or STELA, it cannot deduce TL of individual cells or single molecules. Consequently, it is limited in its detection of TL heterogeneity, a physiologically relevant marker. Moreover, qPCR-based TL data are inherently relative and are not directly comparable between labs. While others have advocated the use of either universal or synthetic oligonucleotide standards to address this (Martin-Ruiz *et al.*, 2015) (O'Callaghan and Fenech, 2011), neither solution has reached widespread implementation as of yet. The incompatibility of data between labs remains an unsolved issue. Furthermore, adoption of MMqPCR by research groups has proven difficult, due to the ambiguity of both its procedure and sources of variability. This report should contribute to mitigating such ambiguity and aid in the broad adoption of this assay. It still remains the most cost-effective method for measuring average TL, the best suited method for large scale studies, with arguably the greatest potential for broad adoption as a

routine clinical tool. A more comprehensive consensus on how to report the usage of MMqPCR and the distribution of universal calibration standards would help further this effort. We expect shared knowledge and experience with MMqPCR to deepen over time, enabling it to become the staple method for precise average TL measurements.

Chapter 3: DYNAMICS OF LEUKOCYTE TELOMERE LENGTH IN HIV-INFECTED AND HIV-UNINFECTED PREGNANT WOMEN: A LONGITUDINAL OBSERVATIONAL STUDY

3.1 Introduction

Women represent more than half of all people living with HIV worldwide (UNAIDS 2017) and approximately 90% of infections in infants and young children occur through MTCT during pregnancy, labour, delivery or breastfeeding (DHHS, 2016). It is well established that cART in pregnancy can greatly reduce this risk. The treatment guidelines recommend to initiate and continue cART throughout pregnancy and thereafter in all pregnant and breastfeeding HIV-infected women, regardless of CD4+ cell count (Money *et al.*, 2014; Rimawi *et al.*, 2016; WHO, 2016). Despite the great success of cART, individuals living with HIV remain at increased risk of premature aging and age-related diseases (Justice and Braithwaite, 2012; Freiberg *et al.*, 2013). Long term data on the safety of cART exposure during pregnancy remain scarce, but unlike in the past when treatment was often initiated in the second trimester, current treatment guidelines mean that HIV-infected women are likely to be on cART throughout conception, and all trimesters of pregnancy.

Cellular aging is associated with many factors, including progressive shortening of telomere whereby in cells that do not express telomerase, telomeres shorten with each cell division (Weischer, *et al.*, 2012; Bhatia-Dey *et al.*, 2016). Shorter LTL has been associated with high mortality (Rode *et al.*, 2015) and increased risk of cardiovascular disease (Zhu *et al.*, 2011). Telomerase, the enzyme complex responsible for the replication of telomeric DNA during cell

division is expressed in germ cells, embryonic and adult tissue stem cells (Choudhary *et al.*, 2012), placenta (Izutsu *et al.*, 1998), hematopoietic stem cells (Greenwood and Lansdorp, 2003) and activated lymphocytes (Sheng *et al.*, 2003), where its activity prevents telomere shortening (Collins and Mitchell, 2002). In most somatic tissues, telomerase is not expressed, and telomeres progressively shorten over time. When telomeres reach a critically short length, cell senescence or death is induced (Kim *et al*., 1994). Some studies have suggested possible links between telomere length/telomerase and reproductive health, including fertility and pre-term delivery (Liu *et al.*, 2002; Antunes *et al.*, 2015; Smeets *et al.*, 2015). In the context of HIV, both the infection and its treatment could modulate telomere attrition through a number of pathways, and contribute to the apparent premature aging reported in people living with HIV (Pace and Leaf, 1995; Tchirkov and Lansdorp, 2003; Epel *et al.*, 2004; Franzese *et al.*, 2007; Justice, 2010). For example, HIV-induced chronic immune activation, inflammation and oxidative stress, HIV protein down- modulation of telomerase activity (Reynoso *et al.*, 2006; Franzese *et al.*, 2007), and NRTI inhibition of telomerase RT (Murakami *et al.*, 1999; Yamaguchi *et al.*, 2001; Datta *et al.*, 2006; Liu *et al.*, 2007; Gillis *et al.*, 2008; Hukezalie *et al.*, 2012; Leeansyah *et al.*, 2013) could accelerate telomere attrition. In addition, several antiretroviral agents can induce oxidative stress (Lagathu *et al.*, 2007; Caron *et al.*, 2008; Kline and Sutliff, 2008), which could also affect LTL (Wolkowitz *et al.*, 2011). Although several studies have associated shorter LTL with HIV infection, an association with exposure to cART exposure *in vivo* has not been clearly demonstrated. In fact, some studies suggest that cART may exert an overall beneficial effect on telomeres (Côté *et al.*, 2012; Imam *et al.*, 2012). Women have a slower LTL attrition than men but nothing is known about how LTL may be affected by pregnancy. Therefore, I examined LTL dynamics over the course of pregnancy in HIV-infected and HIV-uninfected women, explored

predictors of LTL, including the effect of cART itself. I hypothesized that HIV-infected pregnant women would have shorter LTL than HIV-uninfected women and that cART would modulate LTL.

3.2 Materials and Methods

3.2.1 Study Participants

In this prospective longitudinal observational study of HIV-infected and HIV-uninfected pregnant women, participants were enrolled in two consecutive cohorts, namely the Pregnancy cohort and the Children and Women: Antiretrovirals and Markers of Aging (CARMA) cohort in Vancouver, Canada. From December 2004 to March 2009, 133 eligible HIV-infected and HIV-uninfected pregnant women were invited to participate in the Pregnancy cohort and 125 (94%) provided written consent. Similarly, 90 HIV-infected and HIV-uninfected pregnant women were invited to participate in CARMA cohort (from January 2009 to April 2012) and 80 (89%) provided written consent.

These cohorts, funded through two sequential Canadian Institute of Health Research (CIHR) grants, had identical inclusion/exclusion criteria, highly similar protocols for data collection and sample processing, and were led by the same investigators. Inclusion criteria for both HIV-infected and HIV-uninfected women were being pregnant at any age with a known HIV status. Of note, the original inclusion criteria for all women was being 19 years of age or older. However, an amendment was sought and obtained to include younger pregnant participants in the study. For HIV-infected participants, be taking or be willing to take ARVs during pregnancy was the other inclusion criteria. Exclusion criteria included the inability to provide informed consent (language barriers) or to participate in the study (health or social crisis).

HIV-infected participants were recruited exclusively from the Oak Tree Clinic at British Columbia Women's Hospital. HIV-uninfected participants were recruited through a variety of means, including word of mouth and advertisements posted in strategic areas in Vancouver, to promote enrollment of controls with sociodemographic characteristics similar to those of our HIV-infected participants. A $20 honorarium was given to study participants at each visit.

Enrollment in the study usually took place in the first trimester and participants provided biological specimens at three visits during pregnancy. The first visit (A) took place between 13 and 23 weeks of gestation. The second (B) and third (C) visits were at >23-30, and >30-40 weeks of gestation, respectively. In the case of HIV-infected participants, additional visits (with specimen collection) took place at the time of delivery (visit Del) and post-partum, usually between 6 and 8 weeks (visit P-P).

All specimens were stored at -80°C until DNA extraction. In cases of repeat pregnancies, only the first pregnancy was included in this study. Demographic, clinical, and substance use data were collected at each visit. Table 3.1 describes the number of available blood specimens at each study visit.

Table 3.1. Available blood specimens at each study visit. Visits

Visits	**HIV-infected women (n=64)**	**HIV-uninfected women (n=41)**
Pregnancy visits (A, B, and C)	64 (100)[a]	41 (100)
Del	55 (86)	Not available
P-P	59 (92)	Not available

Data are presented as n (%) of available specimens. Abbreviations: Del; Delivery, P-P; post-partum; [a]Blood specimens collected at delivery visit were used in the analyses for two HIV-infected women for whom no visit C blood specimen was collected. The weeks of gestation at delivery for one woman was 32 and for the other one was 40, which fell within the visit C range.

3.2.2 Description of Variables

Age, ethnicity, income, and substance use history (smoking, alcohol, and illicit drugs) were obtained from the participants through self-report. Substance use at visit was coded as a yes/no and was defined as self-reported use of substance(s) at ≥3 visits during pregnancy inclusive of the period prior to delivery. Non-users were all those self-reporting use at <3 visits during pregnancy; only 7 women reported smoking at only 1 or 2 visits. Smoking was defined as tobacco and/or marijuana consumption. The two were highly collinear as 89% of marijuana users also used tobacco and for this reason, I elected to combine both in the smoking variable.

Most cohort studies collect and use self-report data for smoking. However, due to stigma associated with smoking during pregnancy, there is a risk that the behavior may be underreported, leading to study biases. In another study (Saberi *et al*., 2017a), I examined the reliability of self-reported smoking (tobacco use) during pregnancy in our cohorts by measuring plasma cotinine concentration and determining the relationship between the two measures. I found excellent concordance (91%) between plasma cotinine and smoking self-report, indicating that the smoking data are reliable as a surrogate for tobacco exposure. Illicit drugs include heroin, cocaine, opioids, amphetamines, benzodiazepenes and/or 3, 4-methylenedioxy-methamphetamine (MDMA). Smoking and use of illicit drug were collinear and the all women self-reporting using illicit drugs also smoked throughout pregnancy.

History of HCV infection was defined as self-report of HCV+ status and/or a lab test result. The HCV antibody tests were part of early pregnancy testing and results were available for the majority of HIV-infected participants through their medical charts.

For HIV-infected participants, date of HIV diagnosis, CD4+ nadir, CD4+ count at visit, HIV pVL at visit, cART status at visit (on *vs.* off), being antiretroviral therapy naïve, having

conceived on cART, duration of cART in pregnancy (weeks), and type of cART regimens during pregnancy were obtained. cART regimens were categorized as containing a ritonavir boosted PI (PI/r) or not. In case of missing CD4+ count or HIV pVL at pregnancy visits, I imputed the data. When the missing value was for a visit between 2 other visits (i.e. visit B, or Visit C), I used the average of the 2 other available values. To impute the data for visit A, I carried backward the next available value (values from visit B).

Pre-term birth is defined as childbirth occurring at gestational age (GA) less than 37 weeks (WHO, 2016). A small for gestational age (SGA) have birth weights below the 10th percentile for an infant population of the same GA and sex (Kramer *et al.*, 2001). In this study, an SGA infant was calculated according to the British Columbia statistics provided by perinatal service BC.

3.2.3 Relative LTL Measurement

WB DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) on the QIAcube, according to the manufacturer's "Blood and Body Fluid" protocol with the following modifications: 0.1 mL of blood was diluted 1:1 with 0.1 mL phosphate buffered saline prior to extraction, and the DNA was eluted into a final volume of 0.1 mL of buffer AE (Qiagen). Relative LTL were measured by a monochromatic multiplex qPCR assay modified from (Cawthon, 2009) and described in detail previously in chapter two (Hsieh and Saberi *et al.*, 2016). The relative LTL value were expressed as the average of the duplicate T*1000/S ratios (these were multiplied by 2.33 to calibrate according to lymphocyte TL values previously obtained by Flow-FISH (Imam *et al.*, 2012). The LTL assay's inter-run coefficient of variation was under 4% (n=26 runs).

3.2.4 Statistical Analyses

Only study participants who attended all three planned study visits during pregnancy were included in the analyses. Comparisons between groups were done using Fisher's exact test for categorical variables, and Student t-test or Mann-Whitney test for continuous variables using XLSTAT version 2012.6.08 (Addinsoft, NY, USA).

For the longitudinal analyses of the three pregnancy visits (A, B, and C), multivariate analysis of variance (MANOVA) models were performed to explore the relationship(s) between LTL and the following explanatory variables: maternal age, race/ethnicity, income, HIV status, history of HCV infection, substance use throughout pregnancy, weeks of gestation at visit, pre-term delivery (GA<37 weeks), as well as cohort (CARMA *vs*. Pregnancy). The same set of analyses was performed on the entire study sample, as well as within the HIV-infected and HIV-uninfected groups separately. Among the HIV-infected women, in addition to the variables listed above, HIV specific variables including CD4+ cell count at visit, HIV pVL at visit (detectable *vs.* undetectable <50 copies/mL), cART status at visit (on *vs*. off) at visit, and type of cART regimen received during pregnancy (PI/r *vs*. other regimens). Variables univariately associated with LTL with $p \leq 0.1$ were included in multivariable MANOVA models with LTL as the dependent variable. Independent categorical variables with levels populated with less than 5 observations, were omitted from the multivariable analyses, All MANOVA models were performed using JMP software, v. 12.2.0 (SAS Institute, NC, USA).

3.3 Results

3.3.1 Study Participants

The original participation rate in the cohorts was high (>90%). Of note, among the 251 pregnancies in 223 women, after excluding repeat pregnancies and participants who missed one or more study visits in pregnancy, a total of 105 women (64 HIV-infected and 41 HIV-uninfected) were included in this study. A flow-chart of the participant enrollment process and inclusion for this study is presented in Figure 3.1

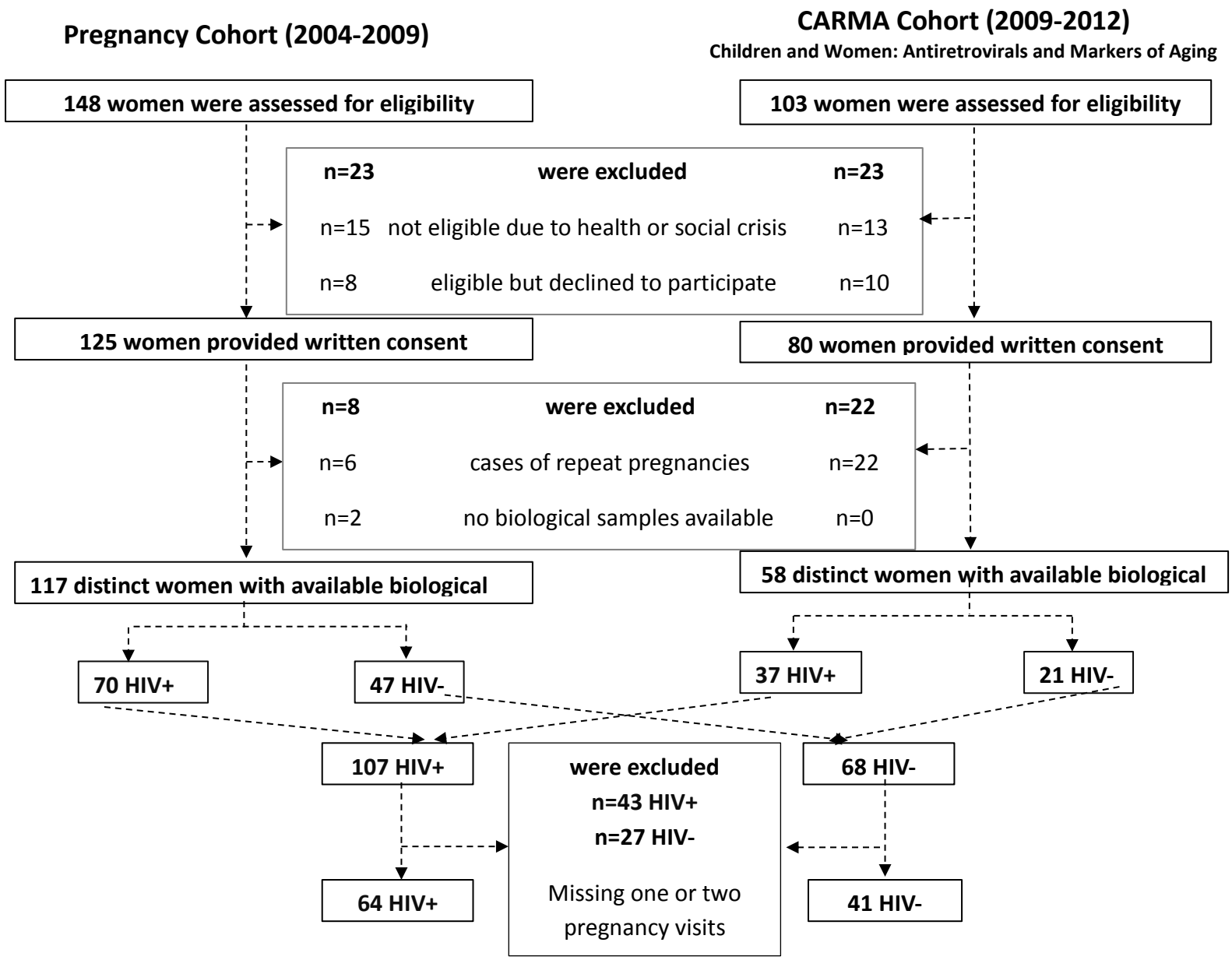

Figure 3.1. Schematic of study participants.

Generally, there were few differences between women who were included *vs*. excluded in this study. HIV-infected women who were included had longer duration of HIV infection and longer exposure to cART during the current pregnancy. HIV-uninfected women who were included were less likely to have a low income, and to smoke throughout pregnancy. The detailed comparisons between included and excluded women are presented in Tables 3.2 & 3.3. Importantly, there was no significant difference between women who were included *vs*. those who were excluded with respect to LTL at each visit.

Table 3.2. Comparison of demographic, clinical, and environmental characteristics between HIV-infected women who were included in and excluded from the analyses.

Characteristics	**HIV-infected women included (n=64)**	**HIV-infected women excluded (n=43)**	**P value**
Maternal age at delivery (years)	31 ± 6 (17-41)	31 ± 5 (18-45)	0.76
Weeks of gestation at visit			
A (n=64, 14)	19 ± 2 (14-23)	20 ± 2 (16-23)	0.14
B (n=64, 30)	26 ± 2 (23-30)	27 ± 2 (23-31)	0.13
C (n=62, 31)	34 ± 2 (30-37)	34 ± 2 (31-38)	**0.04**
Del (n=55, 26)	38 ± 2 (32-41)	39 ± 2 (31-41)	0.34
GA at delivery (weeks)	38 ± 2 (32-42)	38 ± 3 (31-41)	0.77
Pre-term delivery (GA<37 weeks)	12 (19)	9 (21)	0.78
Race/Ethnicity (n=64, 42)			0.23
Indigenous/First Nations	23 (36)	11 (26)	
Black/African Canadians	11 (17)	6 (14)	
White/ Caucasian	18 (28)	20 (48)	
Asian/Other	12 (19)	5 (12)	
Income <$15,000/year (n=64, 41)	33 (52)	22 (54)	0.83
History of HCV infection	25 (39)	16 (37)	0.85
Substance use throughout pregnancy[a]			

Characteristics	HIV-infected women included (n=64)	HIV-infected women excluded (n=43)	P value
Smoking[b] (n=64, 37)	33 (52)	15 (41)	0.28
Illicit drug[c] (n=64, 35)	9 (14)	4 (11)	0.71
Alcohol (n=64, 35)	3 (5)	2 (6)	0.82
HIV-specific characteristics			
Duration of HIV infection at delivery (years)	6.1 ± 4.5 (0.4-19.2)	4.4 ± 4.3 (0.2-17.1)	**0.03**
Age at HIV diagnosis (years)	25 ± 6 (2-36)	26 ± 5 (15-37)	0.28
CD4+ nadir (cells/µL)	288 ± 195 (10-910)	311 ± 210 (20-730)	0.67
Log Highest HIV pVL in pregnancy (n=60, 41)	2.8 ± 1.1 (1.69-5.2)	3.0 ± 1.1 (1.6-5.0)	0.52
Detectable HIV pVL at delivery (>50 copies/ml) (n=45, 20)	8 (18)	2 (10)	0.32
cART exposure during pregnancy (weeks) (n=64, 40)	25.1 ± 10.2 (0.3-41.7)	20.5 ± 10.8 (1.4-41.1)	**0.03**
ART naïve pre-pregnancy	23 (36)	22 (51)	0.12
Conceived on cART	19 (30)	9 (41)	0.31
Received ritonavir-boosted PI during pregnancy	39 (61)	27 (63)	0.85
LTL at Visit			
A (n=64, 13)	7.2 ± 0.8 (5.7-9.2)	6.8 ± 1.3 (4.6-9.0)	0.19
B (n=64, 29)	7.4 ± 0.9 (5.0-10.0)	7.7 ± 1.1 (5.1-11.1)	0.14
C (n=64, 31)	7.4 ± 0.9 (5.6-9.8)	7.8 ± 1.0 (6.0-10.4)	0.07
Del (n=55, 26)	7.2 ± 1.1 (5.5-11.0)	7.6 ± 0.9 (5.4-9.2)	0.07
P-P (n=59, 30)	7.2 ± 0.8 (5.6-9.6)	7.5 ± 1.0 (5.9-10.2)	0.20

Data are presented as mean ± 1 SD (range) or n (% of total included or excluded); unless otherwise indicated. Abbreviations: Del, delivery; GA, gestational age; HCV, Hepatitis C Virus; CD4+; cluster of differentiation; pVL, plasma viral load; cART, combination antiretroviral therapy; LTL, Leukocyte Telomere Length; P-P, Post-Partum. [a]Substance use throughout pregnancy is defined as self-reported use of substance at ≥3 visits during pregnancy inclusive of the period prior to delivery. [b]Smoking includes tobacco and/or marijuana use throughout pregnancy. [c]Illicit drug includes heroin, cocaine, opioids, amphetamines, benzodiazepenes, and/or 3, 4 methylenedioxy-methamphetamine (MDMA).

Table 3.3. Comparison of demographic, clinical, and environmental characteristics between HIV-uninfected women who were included in and excluded from the analyses.

Characteristics	HIV-uninfected women included (n=41)	HIV-uninfected women excluded (n=27)	P value
Maternal age at delivery (years)	31 ± 5 (21-41)	31 ± 5 (21-43)	0.63
Weeks of gestation at visit			
A (n=41, 13)	19 ± 2 (13-22)	19 ± 2 (16-23)	0.37
B (n=41, 17)	26 ± 1 (24-30)	27 ± 2 (23-30)	0.50
C (n=41, 19)	35 ± 2 (32-40)	34 ± 2 (32-38)	0.49
GA at delivery (weeks)	40 ± 2 (35-42)	38 ± 3 (29-41)	0.20
Pre-term delivery (GA<37 weeks)	5 (12)	5 (19)	0.47
Race/Ethnicity			0.08
Indigenous/First Nations	3 (7)	7 (26)	
Black/African Canadians	0 (0)	1 (4)	
White/ Caucasian	31 (76)	14 (52)	
Asian/Other	7 (17)	5 (18)	
Income <$15,000/year	11 (27)	17 (63)	**0.003**
History of HCV infection	2 (5)	5 (19)	0.07
Substance use throughout pregnancy (n=41, n=22)[a]			
Smoking[b]	10 (24)	13 (59)	**0.04**
Illicit drug[c]	4 (10)	6 (27)	0.16
Alcohol	5 (12)	2 (9)	0.52
LTL at Visit			
A (n=41, 14)	7.6 ± 0.9 (6.0-10.6)	7.4 ± 1.5 (5.8-10.2)	0.54
B (n=41, 18)	7.6 ± 0.9 (6.0-9.9)	7.7 ± 1.2 (6.1-10.2)	0.63
C (n=41, 19)	7.7 ± 0.9 (5.9-10.7)	7.9 ± 0.9 (6.5-9.9)	0.54

Data are presented as mean ± 1 SD (range) or n (% of total included or excluded); unless otherwise indicated. Abbreviations: Del, delivery; GA, gestational age; HCV, Hepatitis C Virus; LTL, Leukocyte Telomere Length.[a] Substance use throughout pregnancy is defined as self-reported use of substance at ≥3 visits during pregnancy inclusive of the period prior to delivery. [b]Smoking includes tobacco and/or marijuana use throughout pregnancy. [c]Illicit drug includes heroin, cocaine, opioids, amphetamines, benzodiazepenes and/or 3, 4-methylenedioxy-methamphetamine (MDMA).

Table 3.4 presents the demographic, clinical, and environmental characteristics of the study participants included in the longitudinal analysis. The two groups were similar with respect to age, mode of delivery, weeks of gestation at first and second study visit, and delivery visit, rate of pre-term delivery, and rate of infant small for gestational age. However, race/ethnicity was different between the two groups, with no Black/African Canadians and significantly fewer Indigenous/First Nations in the HIV-uninfected group. HIV-infected women were also more likely to have a history of HCV infection, and to have a low income compared to the HIV-uninfected group. Both groups showed similar rates of illicit drug and alcohol use throughout pregnancy, but HIV-infected women were more likely to smoke throughout pregnancy than HIV-uninfected women. The rates of substance use at each visit are presented in Figure 3.2.

Table 3.4. Demographic, clinical and environmental characteristics of study participants.

Characteristics	**All women (n=105)**	**HIV-infected women (n=64)**	**HIV-uninfected women (n=41)**	**P value**
Maternal age at delivery (years)	31 ± 6 (17-41)	31 ± 6 (17-41)	32 ± 5 (21-41)	0.45
Mode of delivery				
Vaginal	74 (70)	41 (64)	33 (80)	0.52
Non-emergency C-section	8 (8)	6 (9)	2 (5)	
Emergency C-section[a]	23 (22)	17 (27)	6 (15)	
Weeks of gestation at visit				
A (n=105, 64, 41)	19 ± 2 (13-23)	19 ± 2 (14-23)	19 ± 2 (13-22)	0.35
B (n=105, 64, 41)	26 ± 2 (23-30)	26 ± 2 (23-30)	26 ± 1 (24-30)	0.71
C (n=103, 62, 41)	34 ± 2 (30-40)	34 ± 2 (30-37)	35 ± 2 (32-40)	0.02
Del (n=55, 55, n/a)	38 ± 2 (32-41)	38 ± 2 (32-41)	n/a	---
GA at delivery (weeks) (n=105, 64, 41)	39 ± 2 (32-42)	38 ± 2 (32-42)	39 ± 2 (35-42)	0.17

Characteristics	All women (n=105)	HIV-infected women (n=64)	HIV-uninfected women (n=41)	P value
Pre-term delivery (<37 weeks)	17 (16)	12 (19)	5 (12)	0.37
Infant SGA[b] **(n=103, 62, 41)**	15 (15)	11 (18)	4 (10)	0.27
Race/Ethnicity				<0.0001
Indigenous/First Nations	26 (25)	23 (36)	3 (7)	
Black/African Canadians	11 (10)	11 (17)	0 (0)	
White/ Caucasian	49 (47)	18 (28)	31 (76)	
Asian/Other	19 (18)	12 (19)	7 (17)	
Income <$15,000/year	44 (42)	33 (52)	11 (27)	0.01
History of HCV infection[c]	27 (26)	25 (40)	2 (5)	<0.0001
HCV+ Antibody (n=77, 60, 17)	25 (32)	23 (38)	2 (12)	0.04
Substance use throughout pregnancy[d]				
Smoking[e]	43 (41)	33 (52)	10 (24)	0.006
Illicit drug[f]	13 (12)	9 (14)	4 (10)	0.51
Alcohol	8 (8)	3 (5)	5 (12)	0.16
HIV-specific characteristics[g]				
Duration of HIV infection at delivery (years)	---	6.1 ± 4.5 (0.4-19.2)	---	---
Age at HIV diagnosis (years)	---	25 ± 6 (2-36)	---	---
CD4+ nadir (cells/µL)	---	288 ± 195 (10-910)	---	---
Log Highest HIV pVL in pregnancy	---	2.7 ± 1.1 (1.6-5.2)	---	---
Detectable HIV pVL at visit C (>50 copies/ml) (n=63)		12 (19)		
Detectable HIV pVL at delivery (>50 copies/ml) (n=45)	---	8 (18)	---	---
cART exposure during pregnancy (weeks)	---	25.1 ± 10.2 (0.3-41.7)	---	---
ART naïve pre-pregnancy	---	23 (36)	---	---
Conceived on cART	---	19 (30)	---	---
On cART at visit:				

Characteristics	All women (n=105)	HIV-infected women (n=64)	HIV-uninfected women (n=41)	P value
A (n=64)		34 (53)		
B (n=64)		59 (92)		
C (n=62)[h]		62 (100)		
Del (n= 55)		55 (100)		
P-P (n= 59)		38 (64)		
Received ritonavir-boosted PI during pregnancy	---	39 (61)	---	---

Data are presented as mean ± 1 SD (range) or n (% of total) unless otherwise indicated. Abbreviations: GA, gestational age; SGA; small for gestational age, HCV, Hepatitis Virus, CD4+; cluster of differentiation 4; pVL, plasma viral load; cART, combination antiretroviral therapy; PI, protease inhibitor. Characteristics were compared between groups using Fisher's exact test for categorical variables and Student t-test for continuous variables. [a]Mode of C-section is not known for HIV-infected (n=1) and HIV-uninfected (n=1) women and considered as emergency C-section. [b]SGA infants have birth weights below the 10th percentile for an infant population of the same GA and sex. In this study, SGA is calculated according to the British Columbia (BC), Canada statistics provided by perinatal service BC. [c]History of HCV infection was defined as self-report of HCV+ status and/or a lab test result. [d]Substance use throughout pregnancy is defined as self-reported use of substance at ≥3 visits during pregnancy inclusive of the period prior to delivery. [e]Smoking includes tobacco and/or marijuana and 8/9 participants who self-reported marijuana use throughout pregnancy reported tobacco use as well. [f]Illicit drug includes heroin, cocaine, opioids, amphetamines, benzodiazepenes and/or 3, 4-methylenedioxy-methamphetamine (MDMA). [g]Data was imputed for a total of 6 HIV-infected women: CD4+ count and HIV pVL at visit A (n=2), visit C (n=1), CD4+ count (n=2) and pVL (n=1) at visit B. [h]Blood specimens collected at delivery visit were used in the analyses for two HIV-infected women for whom no visit C blood specimen was collected. The weeks of gestation at delivery for one woman was 32 and for the other one was 40, which fell within the visit C range.

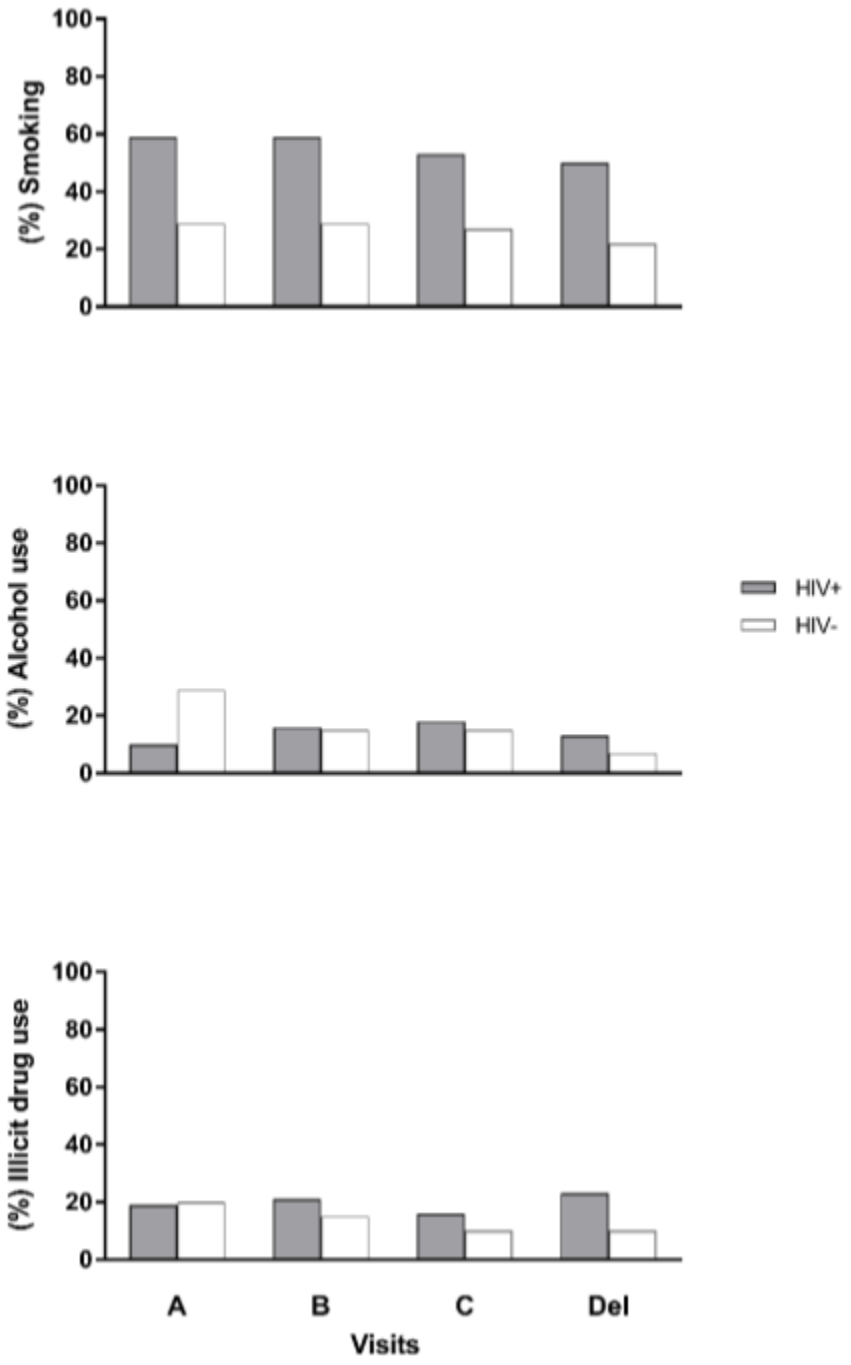

Figure 3.2. Rate of substance use at each visit.

Within the HIV-infected group, the time since HIV diagnosis ranged from very recent (likely diagnosed during routine HIV testing in pregnancy) to almost two decades. Approximately 30% of HIV-infected women conceived on cART, and 36% were cART-naïve

prior to their pregnancy. Additionally, 34% were cART experienced before this pregnancy but were not on cART at the time of conception. All HIV-infected women were on cART by their third visit and 81% of them achieved an undetectable HIV pVL at visit C (Table 3.4). HIV pVL at delivery was only available for 45/64 HIV-infected women and of those, 37 (82%) had an undetectable HIV pVL at delivery. Of note, 14 out of 19 women with unavailable HIV pVL at delivery, achieved undetectable HIV pVL at visit C. Considering available data at visit C and delivery visit, 51 out 59 women for whom pVL data was available (86%) had undetectable HIV pVL at the end of their pregnancy and/or delivery. More than half (64%) continued cART post-

partum. HIV specific characteristics at visit are presented in Figure 3. 3.

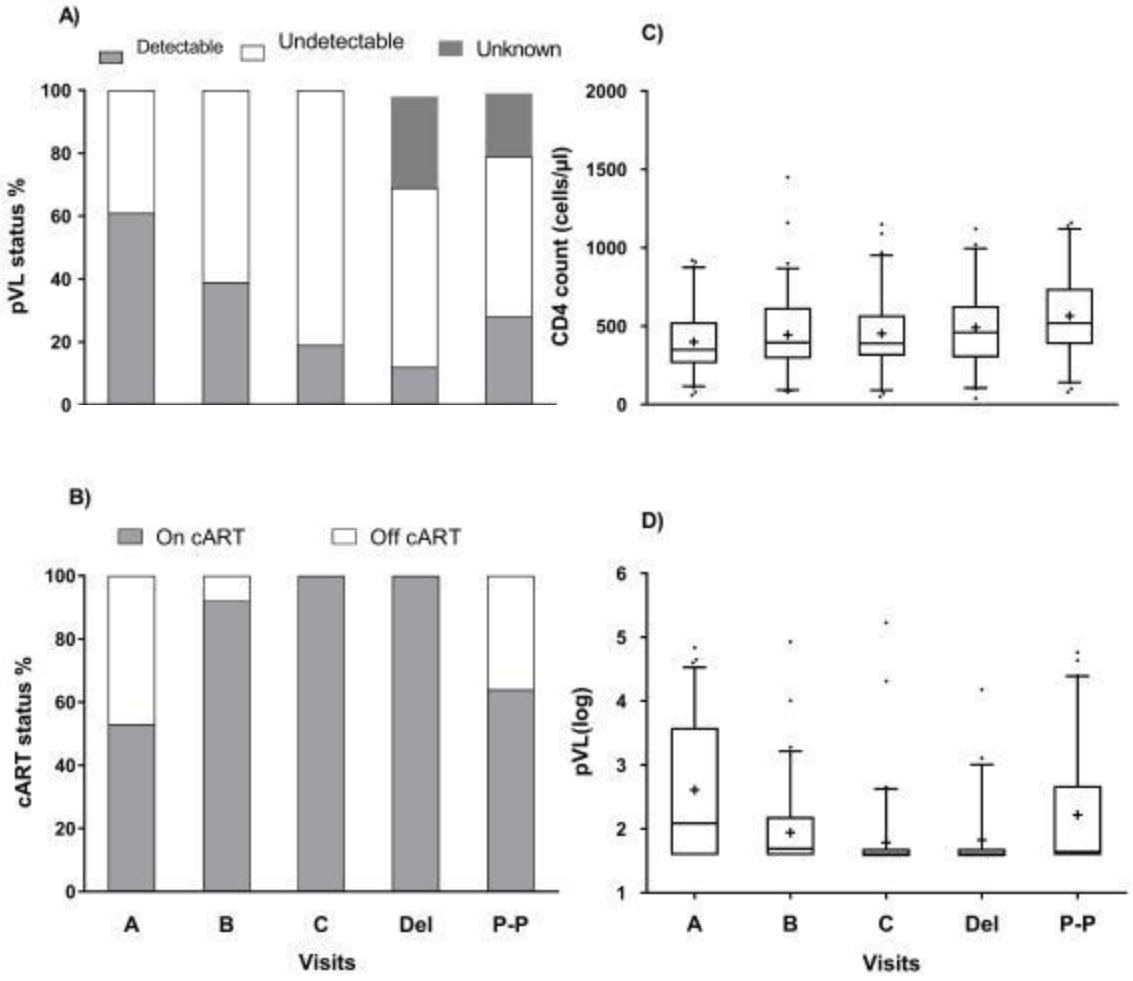

Figure 3.3. HIV specific characteristics at visit.: (A) % with detectable HIV plasma viral load (pVL) (> 50 copies/mL) *vs.* non-detectable, (B) cART status (% on *vs.* off cART), (C) CD4 count (cells/µL), (D) Log pVL.

The cART regimens varied according to treatment guidelines/standard of care at the time, as well as individual circumstances (Table 3.5). Thirty-nine (61%) HIV-infected women received a PI/r-based regimen. The most common NRTI backbone was AZT/3TC (n=47) combined with a PI; LPV/r (n=27) or NFV (n=19).

Table 3.5. Combination antiretroviral therapy (cART) regimens taken during pregnancy by HIV-infected women (n=64).

cART regimens	Base		Back-bone	
Ritonavir-boosted PI (PI/r) regimens (n=39)				
	LPV/r	33	AZT/3TC	27
			ABC/3TC	1
			TDF/FTC	1
			D4T/3TC	2
			AZT/ABC/3TC	1
			AZT/ddI/3TC	1
	ATV/r	6	ABC/3TC	4
			TDF/FTC	2
Other Regimens (n=25)				
	NFV	19	AZT/3TC	18
			D4T/3TC	1
	NVP	3	AZT/3TC	1
			ABC/3TC	2
	ATV	1	TDF/3TC	1
	RTV	1	AZT/3TC/TDF	1
	---	1	AZT/ABC/3TC	1

PI, protease inhibitors; ABC, abacavir; AZT, zidovudine; 3TC, lamivudine; TDF, tenofovir; ddI, didanosine; d4T, stavudine; FTC, emtricitabine; NVP, nevirapine; NFV, nelfinavir; LPV, lopinavir; ATV, atazanavir; RTV, ritonavir; LPV/r, ritonavir-boosted lopinavir; ATV/r, ritonavir-boosted atazanavir

3.3.2 Univariable Association with LTL

In the univariable MANOVA analyses of all women (Table 3.6), HIV+ status, history of HCV infection, and smoking throughout pregnancy were significantly associated with shorter LTL. All other variables considered, including maternal age, weeks of gestation at visit, pre-term delivery, race/ethnicity, income, cohort, alcohol, and substance use (or use of substances of addiction) throughout pregnancy showed no association with LTL. Upon examining variables within subjects, a significant interaction was noted between maternal age and weeks of gestation with respect to LTL among all women ($p=0.002$) and HIV-infected women ($p=0.02$).

Table 3.6. MANOVA models investigating the univariable association of predictors that may affect LTL in all women and dichotomized d by HIV status.

Predictors	All women (n=105)			HIV-infected women (n=64)			HIV-uninfected women (n=41)		
	β	95%CI	P Value	β	95% CI	P Value	β	95%CI	P Value
Maternal age at delivery (years)	0.02	-0.07 – 0.11	0.66	0.06	-0.04 – 0.16	0.25	-0.09	-0.25 – 0.07	0.27
Maternal age at delivery*weeks of gestation	-0.02	-0.03 – -0.01	**0.002**	-0.01	-0.02 – -0.00	**0.02**	-0.03	-0.07 – 0.01	0.08
HIV status (yes *vs.* no)	-0.50	-0.01 – -0.99	**0.04**					---	---
Weeks of gestation at visit									
A	-0.05	-0.13 – 0.04	0.26	0.02	0.08 – 0.12	0.71	-0.19	-0.33 – -0.05	**0.008**
B	0.10	-0.02 – 0.22	0.10	0.09	-0.06 – 0.24	0.24	0.09	-0.13 – 0.30	0.42
C	0.02	-0.08 – 0.12	0.12	0.01	-0.12 – 0.15	0.82	-0.04	-0.23 – 0.15	0.67
Pre-term delivery (GA<37 weeks)	-0.28	-0.95 – 0.37	0.39	0.45	-1.24 – 0.32	0.24	-0.10	-1.32 – 1.12	0.87
Race/Ethnicity (Ref: Caucasian)									
Indigenous/First Nations	-0.62	-1.51 – 0.26	0.37	-0.51	-1.44 – 0.42	0.08	0.12	-2.00 – 2.24	0.96
Black/African Canadians	0.00	-1.20 – 1.19		0.27	-0.91 – 1.46		---	---	
Asian/other	0.71	-0.26 – 1.69		1.22	0.08 – 2.37		-0.20	-1.87 – 1.47	
Income <$15,000/year	-0.19	-0.68 – 0.30	0.44	-0.21	-0.82 – 0.40	0.49	0.20	-0.70 – 1.10	0.66
History of HCV infection[a] (yes *vs.* no)	-0.54	-1.09 – 0.0	0.05	-0.24	-0.87 – 0.39	0.45	-1.56	-3.34 – 0.23	0.09

Predictors	All women (n=105)			HIV-infected women (n=64)			HIV-uninfected women (n=41)		
	β	95%CI	P Value	β	95% CI	P Value	β	95%CI	P Value
Substance use throughout pregnancy[b] (yes vs. no)									
Smoking[c]	-0.68	-1.56 – -0.20	**0.006**	-0.70	-1.29 – -0.11	0.02	-0.34	-1.27 – 0.58	0.45
Illicit drug[d]	0.09	-0.65 – 0.83	0.80	0.08	-0.80 – 0.97	0.85	0.26	-1.08 – 1.60	0.70
Alcohol	0.43	-0.48 – 1.34	0.35	1.36	-0.05 – 2.77	0.06	-0.38	-1.59 – 0.83	0.53
Cohort (CARMA *vs.* Pregnancy)	-0.28	-0.80 – 0.24	0.24	-0.75	-1.36 – -0.14	**0.02**	0.82	-0.07 – 1.71	0.07
Received ritonavir boosted PI during pregnancy	---	---	---	-0.70	-1.31 – -0.09	**0.03**	---	---	---
CD4+ (cells/µL) at visit									
A	---	---	---	0.00	-0.00 – 0.00	0.56	---	---	---
B	---	---	---	-0.00	-0.00 – 0.00	0.61	---	---	---
C	---	---	---	0.00	-0.00 – 0.00	0.78	---	---	---
Detectable HIV pVL at visit (>50 copies/ml)									
A	---	---	---	-0.10	-0.30 – 0.10	0.33	---	---	---
B	---	---	---	-0.13	-0.37 – 0.11	0.29	---	---	---
C	---	---	---	-0.19	-0.48 – 0.10	0.20	---	---	---
cART status at visit (Ref: on cART)									
A	---	---	---	0.02	-0.18 – 0.22	0.86	---	---	---
B	---	---	---	-0.20	-0.64 – 0.24	0.37	---	---	---

Abbreviations: GA, gestational age; HCV, Hepatitis C Virus; CD4+, cluster of differentiation 4; pVL, plasma viral load; cART, combination antiretroviral therapy; [a]History of HCV infection was defined as self-report of HCV+ status and/or a lab test result. [b]Substance use throughout pregnancy is defined as self-reported use of substance at ≥3 visits during pregnancy inclusive of the period prior to delivery. [c]Smoking includes tobacco and/or marijuana and 8/9 participants

who self-reported marijuana use throughout pregnancy reported tobacco use as well. [d]Illicit drug includes heroin, cocaine, opioids, amphetamines, benzodiazepenes and/or 3, 4-methylenedioxy-methamphetamine (MDMA).

Figure 3.4 illustrates this statistical interaction whereby longer LTL is seen over time of pregnancy in women younger than 35 years old.

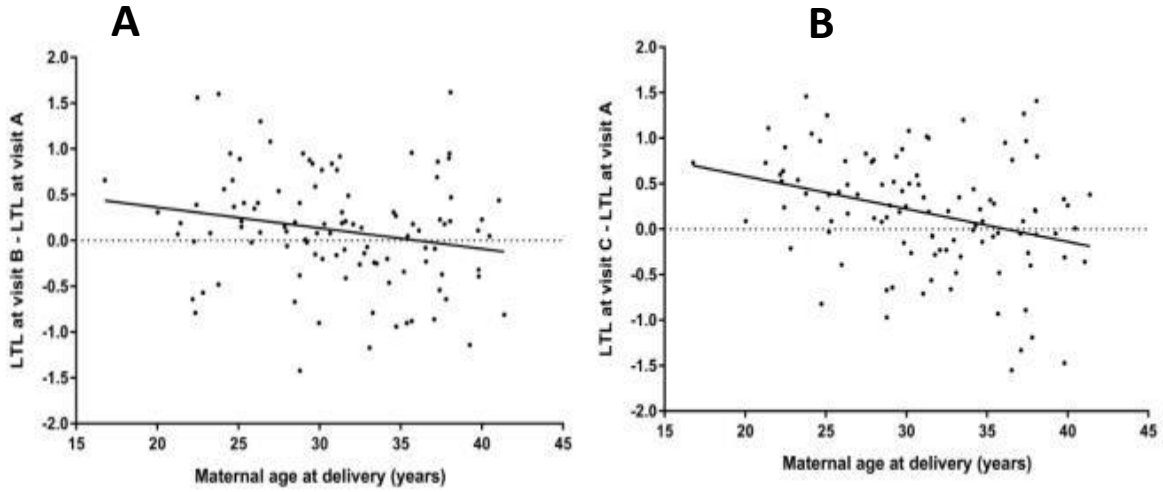

Figure 3.4. Association between maternal age and weeks of gestation. LTL at visit B - LTL at visit A (A) and LTL at visit C - LTL at visit A (B) show that women <35 years old have a net gain in LTL over time (weeks of gestation), demonstrated by a difference >0.

Among all, HIV-infected and HIV-uninfected participants, LTL were correlated significantly across the three pregnancy visits ($p<0.001$). Across the three visits, univariable MANOVA analysis demonstrates that smoking was associated with shorter LTL among all participants and within the HIV-infected group but not the HIV-uninfected group, and the size of its effect was similar for both (β= -0.70 and -0.68). Given that the mean LTL for the entire sample at visit A was 7.4 ± 0.9, this represents a difference of approximately 10% in LTL. Figure 3.5 illustrates LTL at each visit, dichotomized according to smoking status throughout pregnancy, irrespective of HIV status. This unadjusted cross-sectional analysis suggests that smokers (defined as self-reporting smoking at ≥3 visits during pregnancy inclusive of the period prior to delivery) have a pattern of shorter LTL compared to non-smokers (self-reported smoking

at <3 visits during pregnancy) throughout pregnancy (visit A, p=0.001; visit B, p=0.07; visit C, p=0.01). Of note, only 7 women reported smoking at less than 3 visits.

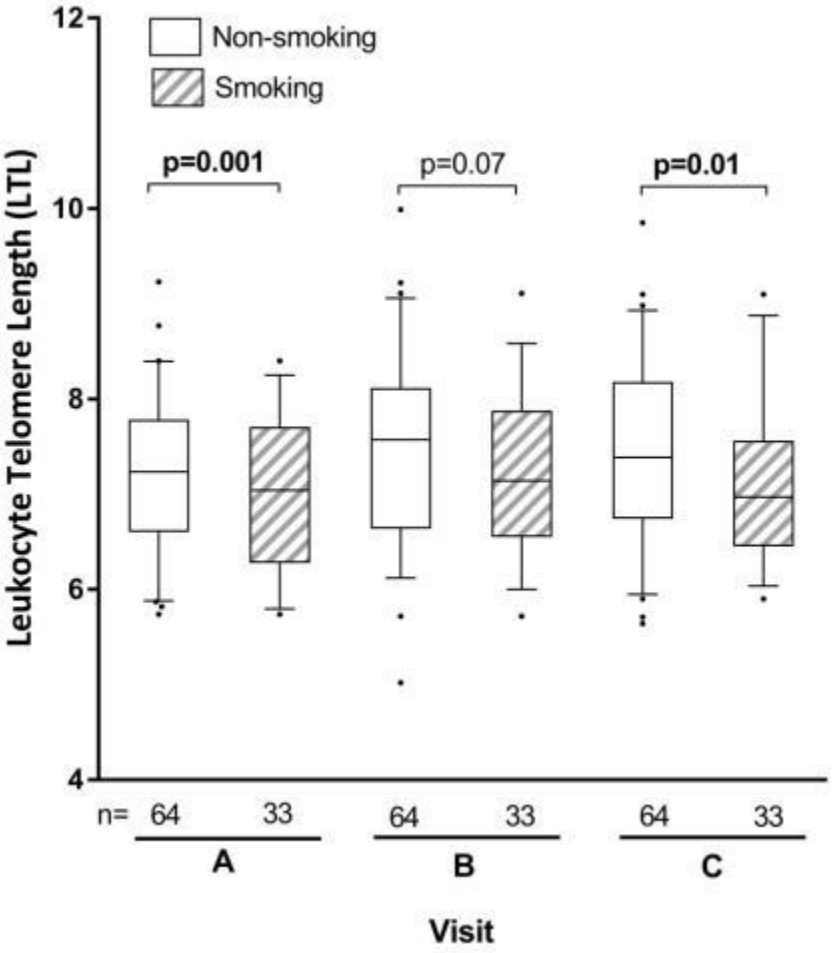

Figure 3.5. Relative **l**eukocyte telomere length (LTL) at visit dichotomized according to smoking status throughout pregnancy. Smoker women self-reported smoking throughout pregnancy (at ≥3 visits during pregnancy inclusive of the period prior to delivery) and non-smoker women self-reported smoking at <3 visits during pregnancy. Comparisons between groups were done using Student's t-test.

Given that these results pointed toward an effect of smoking that was similar in size to my initially hypothesized HIV effect, the study groups were re-examined based on their smoking status. Women who smoked throughout pregnancy were younger, delivered at an earlier

gestational age, were significantly more likely to be HIV-infected, to have a low income, a history of HCV infection, and use substances throughout pregnancy (Table 3.7).

Table 3.7. Demographic and clinical characteristics of study participants separated by smoking status[a]

Characteristics	Smoking throughout pregnancy (n=43)	Non-smoking (n=62)	P value
Maternal age at delivery (years)	30±6 (17- 41)	32±5 (21- 41)	**0.03**
Weeks of gestation at visit (weeks)			
A	19±2 (14-23)	19±2 (13-23)	0.60
B	26±1 (24-30)	26±1 (23-30)	0.09
C (n=42, 62)	34±2 (30-40)	34±1 (31-37)	0.15
Del (n=28, 27)	38±2 (32-41)	39±1 (35- 41)	0.08
GA at delivery (weeks)	38±2 (32-42)	39±2 (35-42)	**0.05**
Pre-term delivery (GA<37 weeks)	9 (21)	8 (13)	0.27
Infant Small for GA[b] (n=42,61)	7 (17)	8 (13)	0.62
Race/Ethnicity			**<0.001**
Indigenous/First Nations	21 (49)	5 (8)	
Black/African Canadians	0 (0)	11 (17)	
White/ Caucasian	19 (44)	30 (48)	
Asian/Other	3 (7)	16 (25)	
Income <$15,000/year	28 (65)	17 (27)	**<0.001**
History of HCV infection[c]	25 (81)	2 (3)	**<0.001**
HIV+ status	33 (77)	31 (50)	**0.006**
Substance use throughout pregnancy[d] (yes *vs.* no)			
Illicit drug[e]	13 (30)	0 (0)	**<0.001**
Alcohol	3 (7)	5 (8)	0.836

Data are presented as mean ± 1 SD (range) or n (% of total) unless otherwise indicated. Abbreviations: GA, gestational age; Del; delivery; HCV, Hepatitis C Virus. [b]Small for GA infants have birth weights below the 10th percentile for infants of the same GA and sex. In this study, small for GA is calculated according to the British Columbia (BC), Canada statistics provided by perinatal service BC[c]. History of HCV infection was defined as self-report of HCV+ status and/or a lab test result.[d] Substance use throughout pregnancy is defined as self-reported use of substance at ≥3 visits during pregnancy inclusive of the period prior to delivery. [e]Illicit drug includes heroin, cocaine, opioids, amphetamines, benzodiazepenes and/or 3, 4-methylenedioxy-methamphetamine (MDMA).

The univariable MANOVA analysis among HIV-infected women suggested that being a CARMA cohort participant and receiving a PI/r regimen during pregnancy were also associated with shorter LTL (Table 3.6). In contrast, for HIV-uninfected women, the univariable MANOVA model showed being a Pregnancy cohort participant as the strongest predictor of shorter LTL ($p=0.07$) apart from the weeks of gestation at visit A. This observation prompted us to examine the makeup of each cohort and the cART type distribution within. As shown in the Table 3.8, collinearity existed between cohort and cART type, whereby HIV-infected women in the CARMA cohort were more likely to receive a PI/r regimen (91%) relative to women enrolled in the earlier Pregnancy cohort (32%) ($p<0.001$); they also had shorter LTL. This difference in cART usage between the two cohorts, mostly driven by change in prescribing practices over time, likely explains the cohort association in HIV-infected participants. Although there was no significant difference between the characteristics of the HIV-uninfected women in the CARMA and Pregnancy cohorts, the rate of smoking in the Pregnancy cohort (29%) was higher than the CARMA cohort rate (10%). Of note, only 24% of the HIV-uninfected women were CARMA cohort participants. No significant relationships were seen between LTL and CD4+ cell counts, detectable HIV pVL, and cART status at visit.

Table 3.8. MANOVA models investigating the univariable association of predictors that may affect LTL in all women and separated by HIV status.

Characteristics	All women (n=105)		P value	HIV-infected women (n=64)		P value	HIV-uninfected women (n=41)		P value
	CARMA (n=33)	Pregnancy (n=72)		CARMA (n=23)	Pregnancy (n=41)		CARMA (n=10)	Pregnancy (n=31)	
Maternal age at delivery (years)	32± 5 (21-40)	31 ± 6 (17-41)	0.61	32 ± 5 (22-40)	30 ± 6 (17-41)	0.30	31 ± 5 (21-36)	32 ± 5 (22-41)	0.48
Pre-term delivery (GA<37 weeks)	4 (12)	13 (18)	0.44	4 (14)	8 (20)	0.83	0 (0)	5 (16)	0.17
Race/Ethnicity									
Indigenous/First Nations	11 (3)	15 (21)	0.48	10 (43)	13 (32)	0.80	1 (10)	2 (6)	0.25
Black/African Canadians	4 (12)	15 (21)		4 (17)	8 (20)		0 (0)	0 (0)	
White/ Caucasian	3 (19)	8 (11)		3 (13)	8 (20)		9 (90)	22 (71)	
Asian/Other	15 (45)	34 (47)		6 (26)	12 (29)		0 (0)	7 (23)	
Income <$15,000/year	15 (45)	29 (40)	0.61	12 (52)	21 (51)	0.94	3 (30)	8 (26)	0.79
History of HCV infection (yes *vs.* no)[a]	12 (36)	15 (21)	0.09	12 (52)	13 (32)	0.11	0 (0)	2 (6)	0.41
Substance use throughout pregnancy[b](yes *vs.* no)									
Smoking[c]	15 (45)	28 (64)	0.52	14 (61)	19 (46)	0.26	1 (10)	9 (29)	0.22
Illicit drug[d]	5 (15)	8 (11)	0.56	5 (22)	4 (10)	0.17	0 (0)	4 (13)	0.23
Alcohol	2 (6)	6 (8)	0.68	1 (4)	2 (5)	0.92	1 (10)	4 (13)	0.81

Characteristics	All women (n=105)		P value	HIV-infected women (n=64)		P value	HIV-uninfected women (n=41)		P value
	CARMA (n=33)	Pregnancy (n=72)		CARMA (n=23)	Pregnancy (n=41)		CARMA (n=10)	Pregnancy (n=31)	
Duration of HIV infection at delivery (years)	---	---	---	6.6 ± 5.2 (0.4-19.2)	5.8 ± 4.1 (0.4-15.3)	0.64	---	---	---
Age at HIV diagnosis (years)	---	---	---	25 ± 7 (3-36)	24 ± 6 (2-35)	0.45	---	---	---
CD4 nadir (cells/mm^3)	---	---	---	285 ± 217 (10-800)	292 ± 184 (20-910)	0.64	---	---	---
Detectable HIV pVL at delivery (>50 copies/ml) (n=29, 16)	---	---	---	5 (38)	3 (11)	0.04	---	---	---
cART exposure during pregnancy (weeks)	---	---	---	25.9 ± 9.9 (8.7-40.1)	24.6 ± 10.6 (0.3-41.7)	0.85	---	---	---
ART naïve pre-pregnancy	---	---	---	9 (39)	14 (34)	0.69	---	---	---
Conceived on cART	---	---	---	6 (26)	13 (23)	0.63	---	---	---
Received ritonavir-boosted PI during pregnancy	---	---	---	21 (91)	13 (32)	**<0.001**	---	---	---

Data are presented as mean ± 1 SD (range) or n (% of total) unless otherwise indicated. Abbreviations: GA, gestational age; HCV, Hepatitis C Virus; CD4+; cluster of differentiation 4; HIV pVL, HIV plasma viral load; cART, combination antiretroviral therapy. [a]History of HCV infection was defined as self-report of HCV+ status and/or a lab test result.[b]Smoking throughout pregnancy is defined as self-reported use of tobacco and/or marijuana at ≥3 visits during pregnancy inclusive of the period prior to delivery. [d]Illicit drug includes heroin, cocaine, opioids, amphetamines, benzodiazepenes and/or 3, 4-methylenedioxy-methamphetamine (MDMA)

In Figure 3.6, LTL is cross-sectionally presented at each visit, dichotomized according to HIV/cART status. As noted above, there is no clear LTL difference between HIV-infected women who were on cART *vs*. off cART at visit A and B. However, HIV-uninfected women had longer LTL at visit A and this difference was attenuated later in pregnancy (Table 3.9). Among HIV-infected participants with a post-partum visit, women who stopped cART had significantly shorter LTL at post-partum compared to visit C while LTL did not change for those who remained on cART (Figure 2B).

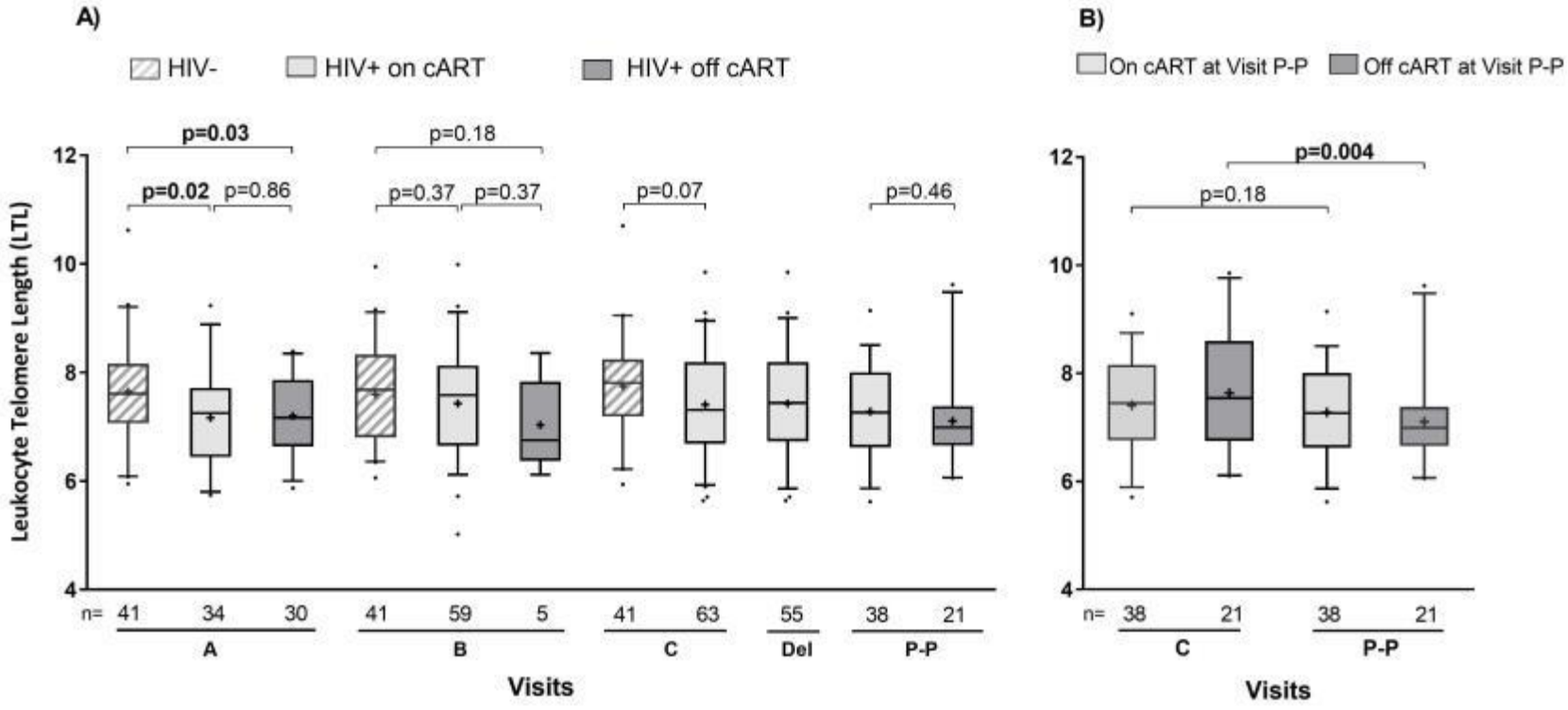

Figure 3.6. Relative leukocyte telomere length (LTL) at visit dichotomized according to HIV/cART status (A) comparisons between groups were done using Student's t-test (B) comparisons within HIV+ women at visit C and P-P were done using paired t-test.

Table 3.9. Leukocyte telomere length (LTL) at visit A, B, and C separated by HIV status and at delivery and post-partum only for HIV-infected women.

LTL	All women (n=105)	HIV-infected women (n=64)	HIV-uninfected women (n=41)	P value
A (n=105, 64, 41)	7.4 ± 0.9 (5.7-10.6)	7.2 ± 0.8 (5.7-9.2)	7.6 ± 0.9 (6.0-10.6)	**0.006**
B (n=105, 64, 41)	7.5 ± 0.9 (5.0-10.0)	7.4 ± 0.9 (5.0-10.0)	7.6 ± 0.9 (6.1-10.0)	0.28
C (n=103, 62, 41)	7.5 ± 0.9 (5.6-10.7)	7.4 ± 0.9 (5.6-9.8)	7.7 ± 0.9 (5.9-10.7)	0.09
Del (n=55)	7.2 ± 1.0 (5.5-10.9)	7.2 ± 1.0 (5.5-10.9)	---	---
P-P (n=59)	7.2 ± 0.8 (5.6-9.6)	7.2 ± 0.8 (5.6-9.6)	---	---

3.3.3 Multivariable Association with LTL

In multivariable MANOVA analyses (Figure 3.7, Table 3.10) the strongest predictor of shorter LTL in the model for all women, and HIV-infected women was the interaction between maternal age and weeks of gestation at visit (p=0.01, β=-0.02 and p=0.01, β=-0.04, respectively). This once again suggests that the phenomenon of apparent longer LTL in younger women associated with pregnancy progression (weeks of gestation), counteracted by increased maternal age that was found in the univariable MANOVA, was still statistically significant in the multivariable model. Smoking throughout pregnancy was the second strongest predictor of shorter LTL among all women (p=0.06), and showed the largest effect size (β= -0.61). However, this association only reached statistical significance in the HIV-infected group (p= 0.04). In addition, having received a PI/r showed an independent association with shorter LTL (p=0.03), and a similar effect size (β= -0.64). For the HIV-uninfected group, none of the possible predictors examined showed an independent association with LTL.

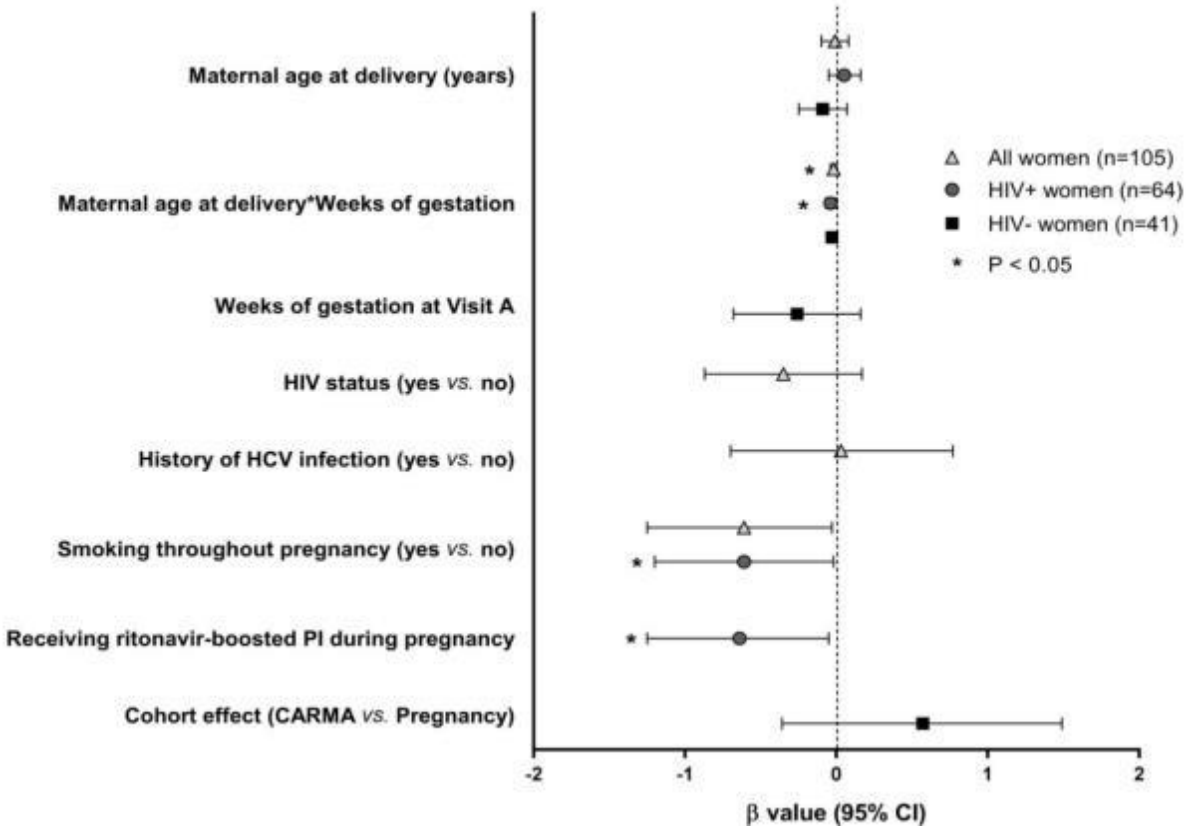

Figure 3.7. Multivariable analyses of the association between various factors and Leukocyte Telomere Length (LTL). MANOVA models were separated by group: all women (light gray triangle), HIV+ women (dark gray circle), and HIV- women (black square). β values and 95% confidence intervals (CI) are shown and negative β values indicate associations with shorter LTL. Abbreviations: HCV, Hepatitis C Virus; PI, Protease inhibitor. [a]History of HCV infection is based on lab results and/or self-report. [b]Smoking throughout pregnancy is defined as self-reported use of tobacco and/or marijuana at ≥3 visits inclusive of the period prior to delivery. Only variable with p≤0.1 univariately are shown.

Table 3.10. Multivariate analyses of the association between various factors and LTL in all participants and separated by HIV status.

Predictors	All women (R^2= 0.19) (n=105)			HIV-infected women (R^2=0.23) (n=64)			HIV-uninfected women (R^2=0.40) (n=41)		
	β Value	**95%CI**	**P Value**	**β Value**	**95%CI**	**P Value**	**β Value**	**95%CI**	**P Value**
Maternal age at delivery (years)	-0.01	-0.10 – 0.08	0.86	0.05	-0.05 – 0.16	0.29	-0.09	-0.25 – 0.07	0.27
Maternal age at delivery*Weeks of gestation	-0.02	-0.04 – -0.00	**0.01**	-0.04	-0.07 – -0.00	**0.02**	-0.03	-0.07 – 0.01	0.12
Weeks of gestation at Visit A	---	---	---	---	---	---	-0.26	-0.68 – 0.16	0.23
HIV status (yes *vs.* no)	-0.35	-0.87 – 0.17	0.19	---	---	---			
History of HCV infection[a]	0.03	-0.70 – 0.77	0.93	---	---	---	---	---	---
Smoking throughout pregnancy[b] (yes *vs.* no)	-0.61	-1.25 – -0.03	0.06	-0.61	-1.20 – -0.02	**0.04**	---	---	---
Received ritonavir-boosted PI during pregnancy	---	---	---	-0.64	-1.25 – -0.05	**0.03**	---	---	---
Cohort (CARMA *vs.* Pregnancy)	---	---	---	---	---	---	0.57	-0.36 – 1.49	0.23

Abbreviations: HCV, Hepatitis C Virus; PI, Protease inhibitor. [a]History of HCV infection was defined as self-report of HCV+ status and/or a lab test result.[b]Smoking throughout pregnancy is defined as self-reported use of tobacco and/or marijuana at ≥3 visits during pregnancy inclusive of the period prior to delivery.

3.4 Discussion

In this study, both the HIV-infected and HIV-uninfected groups showed similar rates of pre-term birth that were higher than those reported in Canada (7.9%) and British Columbia (9.7%) (Canadian Institute for Health Information, 2012). This is in contrast to previous studies, which reported an increased incidence of pre-term birth and SGA infants born to HIV-infected cART-treated women compared to infants born to HIV-uninfected women (Cooper *et al.*, 2002; Town *et al.*, 2007). This is likely explained by the fact that we deliberately enrolled HIV-uninfected participants who shared many non-HIV related risk factors for pre-term delivery with the HIV-infected participants (Robert *et al.*, 2000; Ion, 2015), which is not the case in other cohorts. It is well recognized that, in high resource countries, smoking is highly prevalent among HIV populations (Lifson *et al.*, 2010; Helleberg *et al.*, 2013). In agreement with this, we observed a rate of smoking noticeably higher (43%) than that reported for Canadian pregnant women (17%) (Millar, 2004). This is likely influenced by the socio-economic status and ethnic makeup of our cohort (Wenman *et al.*, 2004). Shorter LTL was not associated with pre-term birth in this study.

My data did not show any association between maternal age and shorter LTL. This may in part be related to the fact that most women were within a narrow age range (25 to 35 years), an age span during which LTL is relatively stable as opposed to early and late life (Lansdorp, 2008).The dynamics of LTL during pregnancy have not been previously studied longitudinally. I demonstrated that, within women, LTL are significantly correlated throughout pregnancy and relatively stable. My data further suggest that relative LTL appeared to be longer in younger women as pregnancy progresses, but this effect wanes as maternal age increases. This may be related to the fact that maternal blood volume increases during pregnancy, inducing new

leukocyte production which may have longer LTL. Age-related differences in leukocyte turnover and new leukocyte generation may in part explain the difference in effect seen in LTL as pregnancy progresses between younger and older women.

In a univariable approach, both living with HIV and smoking throughout pregnancy showed an association with shorter LTL, and of similar effect size. However, in the multivariable model, LTL was most influenced by weeks of gestation. The effect of both HIV and smoking was in the direction of shorter LTL but was not significant. In the multivariable model for HIV-infected women, smoking was independently associated with shorter LTL. Of note, 30% of women who smoked throughout pregnancy also self-reported substance use, while none of the non-smokers did. This implies that the association between smoking and shorter LTL may also be at least partially explained, or confounded, by other substances of addiction. This would be in agreement with previous studies reporting the association between smoking and illicit drugs and shorter LTL, including ones in mothers and their infants (Valdes *et al.*, 2005; Morlá *et al.*, 2006; Song *et al.*, 2010; Yang *et al.*, 2013; Huzen *et al.*, 2014; Müezzinler *et al.*, 2015). Increased oxidative stress, leukocyte turnover, and apoptosis as well as modulation of gene expression have been linked to this effect on LTL (Kovacic and Cooksy, 2005; Huang *et al.*, 2009). The interaction observed between maternal age and weeks of gestation with respect to LTL may also be partially explained by the fact that younger women were more likely to smoke. The latter may also explain their apparent shorter LTL at the first visit. Furthermore, it is well recognized that smoking is stigmatizing, particularly for pregnant women. As pregnancy progresses, it is possible that some participants reduced the intensity of their smoking, possibly positively impacting LTL. Because we did not capture data on smoking intensity, we can only speculate that differences in smoking intensity according to maternal age and/or week of gestation may

play a role here. Smoking is a known risk factor for adverse pregnancy outcomes (Ng and Zelikoff, 2007; Leite *et al.*, 2014) and my findings further support the importance of smoking cessation in pregnancy.

Among HIV-infected participants, factors associated with LTL were the same as those seen in all women, with the addition of cART regimen. Women treated with a PI/r regimen had shorter LTL compared to women who received other regimens. Although *in vitro* and *ex vivo* studies have shown telomerase inhibition by NRTIs (Hukezalie *et al*., 2012; Leeansyah *et al.*, 2013), a study of well-controlled HIV-infected individuals randomized to receive ritonavir-boosted darunavir with or without two NRTIs reported no difference in peripheral blood mononuclear cell (PBMC) TL between groups, nor was it changed upon cessation of the NRTIs (Paintsil *et al.*, 2014). PIs, primarily LPV, have been shown to increase oxidative stress in cell culture models (Lefèvre *et al.*, 2010), something that could play a role here. Increased risk of pre-term delivery has also been associated with PI/r in some studies (Patel *et al*., 2010; Powis *et al.*, 2011; Chen *et al.*, 2012; Sibiude *et al.*, 2012; Kakkar *et al.*, 2015), but other studies reported no difference (Szyld *et al*., 2006; Rudin *et al*., 2011). My study was not powered to examine the relationships with pre-term delivery. The observed association between PI/r and shorter LTL requires further study.

For the HIV-infected group, a post-partum specimen was collected which allowed the within subject comparison between LTL late in pregnancy, when all women were receiving cART, and post-partum, when approximately 48% of women discontinued cART. Among HIV-infected women, the shorter LTL observed post-partum may be related to cART interruption although other factors such as changes in leukocyte count post-partum may also play a role (Chandra *et al.*, 2012).

3.5 Strengths and Limitations

The major strength of this study was the longitudinal design, which allowed insight into the LTL dynamics during pregnancy among HIV-infected cART-treated and HIV-uninfected control women. Furthermore, the HIV-infected and HIV-uninfected groups were well balanced with respect to important factors such as age, illicit drugs and alcohol use throughout pregnancy, reducing the effect of these potential confounders. However, this study also has several limitations. We measured TL in total leukocytes and are not able to address TL change in specific cell subsets. Although it has been shown that the count of various cell subsets varies during pregnancy, their proportion remains largely unchanged (Iwatani *et al.*, 1988). Similarly, cell subset TL in premenopausal women was shown to be highly correlated to that of PBMC TL (Lin *et al.*, 2016). Hormone levels were not measured in this study. I therefore cannot ascertain the possible effect of changes in hormone levels on LTL. Specifically, estrogen increases during pregnancy and decreases post-partum. The hormone has been associated with increased telomerase activity and antioxidant effects (Sack *et al.*, 1994; Römer *et al.*, 1997; Kyo *et al.*, 1999; Massafra *et al.*, 2000; Shin and Lee, 2016). Although I also cannot fully address the physiological change in LTL naturally occurring over the course of pregnancy/delivery/post-partum due to the unavailability of post-partum samples in the HIV-uninfected group, my data could be at least partially explained by expected estrogen changes. Moreover, I could not ascertain whether the decrease in LTL seen post-partum in the HIV-infected women is related to pregnancy ending or is specific to this population. However, my data suggest that staying on cART may protect LTL, possibly through reduced immune activation hence oxidative stress. Finally, the groups were not balanced with respect to ethnic makeup, income, and history of HCV infection, and the latter was not included in the multivariable HIV-uninfected model due to

the low prevalence in our sample. Although I have indicated that the self-report smoking data are robust, the frequency/intensity/timing of smoking was only available for a subset of participants hence was not included. However, based on the participants for whom these details were known, smoking habits did not change noticeably over time. Overall, the HIV-uninfected model explained 40% of the LTL variance, which is higher than for the HIV-infected and all-participants models, suggesting that important yet unidentified HIV-related factors were not considered. This study is not designed to explore the mechanisms behind the reported observations such as the effect of smoking and PI/r on telomere shortening. However, it has been shown that tobacco exposure induces oxidative stress and can damage telomeric DNA (*Asami et al.*, 1996; d'Adda di Fagagna *et al.*, 2003; Opresko *et al.*, 2005; Von Zglinicki, 2002). Similarly, PIs induce premature senescence and oxidative stress in endothelial cells (Lefèvre *et al.*, 2010), something that could be a factor as well. Finally, as for any observational study, these findings should be confirmed in an independent cohort.

3.6 Conclusions

In conclusion, LTL was observed to be relatively stable throughout pregnancy in both HIV-infected and HIV-uninfected women. However, after controlling for important variables, smoking throughout pregnancy and receiving a PI/r were independently associated with shorter LTL among HIV-infected pregnant women. Whether these reflect telomere attrition or redistribution of cellular subsets remains unclear.

Despite my initial hypothesis, cART treatment status during pregnancy does not appear to be a predictor of shorter LTL. However, shorter LTL in post-partum may be partially related to cART interruption. These imply that other factors, such as smoking, and substance use may exert more influence on LTL among HIV-infected women than cART itself. My study further

suggests that LTL in younger women appeared to be longer as pregnancy progresses. I hypothesize that physiological changes during pregnancy as well as potential alterations in environmental factors such as a decrease in intensity of smoking may contribute to this effect.

In this study, there were no significant differences in birth outcomes between HIV-infected and HIV-uninfected women, which is in contrast to several studies reporting an increased incidence of pre-term birth and low birth weight among infants born to HIV-infected cART-treated women compared to infants born to HIV-uninfected women. Of note, the HIV-uninfected control group in this study was well balanced with respect to other risk factors known to be associated with prematurity/low birth weight, including illicit drug use. The rate of smoking and pre-term birth in our study and control groups were noticeably higher than the general population. This may have minimized differences in LTL between HIV-infected and HIV-uninfected groups in this study. This study describes for the first time the dynamics of LTL in pregnancy and although the relationship between maternal LTL and pregnancy outcome is unknown, my results are in accordance with previous studies and highlight the importance of smoking cessation, especially in pregnancy.

Chapter 4: EFFECTS OF PHARMACEUTICAL CONCENTRATIONS OF ANTIRETROVIRAL DRUGS ON MITOCHONDRIAL DNA CONTENT AND DYNAMICS IN CELL CULTURE MODELS

4.1 Introduction

The introduction of ART changed the nature of HIV infection from a terminal disease to a chronic one (Volmink, *et al.*, 2007; Dieffenbach and Fauci, 2011; Siegfried *et al.*, 2011; DHHS, 2016; WHO, 2016). In 2013, the WHO guidelines recommended the initiation of life-long cART earlier in the disease; that is at CD4+ ≤500 cells/µL, for all HIV-infected individuals including children older than five years. These guidelines further recommended immediate initiation of lifelong cART, irrespective of CD4+ cell count or clinical stage, for HIV-infected pregnant women (Doherty *et al.*, 2013). Given this, drug toxicity becomes a major concern that must be investigated to better understand its mechanism(s), in order to help identify and prevent both short and long-term effects.

Mitochondrial toxicity is a major complication that could influence the long-term safety of cART use, and is of special concern for children exposed to ARVs *in utero* and/or postnatally (Blanche *et al*., 1999; Haas, 2000; Côté *et al*., 2008; Crain *et al*., 2010). Early ARVs were implicated in a wide range of complications in HIV-infected ART-treated individuals, including lactic acidosis, hepatic steatosis, neuropathy, myopathy, pancreatitis, and lipodystrophy , many of these involving mitochondrial toxicity (Mussini, and Viganò, 2001; Shikuma and Shiramizu, 2001; Côté *et al*., 2002; Gerschenson and Brinkman, 2004; Garrabou *et al*., 2009). Newer ARVs show reduced acute toxicity but may still exert more subtle long-term mitochondrial toxicity.

Various ARVs may have different effects on mtDNA POLγ and mitochondria (Benbrik *et al*., 1997). It is well established that NRTIs can inhibit mtDNA POLγ (Lee *et al.,* 2003), which can lead to a decrease in mtDNA quantity but is also postulated to affect mtDNA quality (Martin *et al.,* 1994; Lewis and Dalakas, 1995; Walker *et al.*, 2002; Payne *et al.*, 2011; Jitratkosol *et al*., 2012). These phenomena are believed to affect mitochondrial function, although this effect may be sub-clinical, especially with newer ARVs. Importantly, mitochondrial toxicity is not exclusively associated with mtDNA depletion but also involves several other mechanisms, including alterations in OXPHOS enzyme activities, increases in ROS (McComsey and Morrow, 2003; Turchan *et al*., 2003; Apostolova *et al*., 2010), changes in the expression of uncoupling proteins (Pace *et al*., 2003), and changes in autophagy and apoptosis (Badley *et al.*, 2003; Apostolova *et al.*, 2011). It remains unclear whether ARVs that do not overtly inhibit mtDNA POLγ are less toxic as some of them appear to affect mitochondrial function without evidence of mtDNA depletion (Sato, 2007; Abdul-Ghani and DeFronzo, 2008). PIs induce oxidative stress and alter adipocyte insulin resistance through mitochondrial toxicity (Rudich *et al.,* 2005; Ben-Romano *et al*., 2006; Chandra *et al.*, 2009) and NNRTIs increase oxidative stress and release mitochondrial apoptogenic factors (Pilon *et al*., 2002; Apostolova *et al*., 2010). Finally, mitochondrial dysfunction is accelerated by ARVs through the clonal expansion of mtDNA mutations (Payne *et al*., 2011).

Currently, there is no gold standard to measure mitochondrial function in HIV-infected individuals and it is unclear how currently used cART regimens may affect mitochondria. To address this, the present study aimed to: (i) measure changes in mtDNA content in cell culture models following short term exposure to 11 different ARVs, and (ii) investigate longitudinal alterations in mtDNA content and morphology of mitochondria following prolonged exposure to

cART regimens. I focused on ARVs that have been or are extensively used during pregnancy, in North America and/or throughout the world.

4.2 Materials and Methods

4.2.1 Reagents and Drugs

Chemicals including dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), hydrogen peroxide (H_2O_2), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Cell culture reagents and vessels were supplied by Invitrogen (Thermo Fisher Scientific, Waltham, MA, US) and Gibco (Life Technologies, Eugene, OR, US). ARVs were obtained from the National Institute of Health (NIH) AIDS Reagent Program and were dissolved in DMSO, for a 0.1% final concentration in culture except for EFV and LPV at 20 $C_{max,}$ for which the final DMSO concentration is 0.3%. Table 4.1 shows the maximum concentration (C_{max}) of ARVs in plasma that guided the experiments.

Table 4.1. Summary of maximum concentration (C_{max})* of ARVs used in this study

	NRTIs						**NNRTIs**		**PIs**			**InSTI**
ARVs	**d4T**	**AZT**	**3TC**	**FTC**	**TDF**	**ABC**	**EFV**	**NVP**	**NFV**	**LPV**	**RTV**	**RAL**
C_{max} (µM)	2.39	5.20	6.54	7.28	0.47	4.47	12.90	7.51	6.02	15.00	1.23	6.53

NRTIs: Stavudine (d4T), Zidovudine (AZT), Lamivudine (3TC), Emtricitabine (FTC), Tenofovir disoproxil fumarate (TDF), and Abacavir (ABC); NNRTIs: Efavirenz (EFV), Nevirapine (NVP); PIs: Nelfinavir (NFV), Lopinavir (LPV), and Ritonavir (RTV); InSTI: Raltegravir (RAL).
* The values are obtained from US Food & Drug Administration (FDA), https://www.accessdata.fda.gov

4.2.2 Selection of cART Investigated

I aimed to study cART regimens that either have been, or are expected to become used extensively during pregnancy, considering both North American and WHO guidelines. Currently, the first line cART regimens recommended by US guidelines for pregnant women

living with HIV include a backbone of two NRTIs plus a PI/r, or a NNRTI, or an InSTI. The preferred NRTIs are ABC/3TC or TDF/FTC, both of which are available as fixed-dose drug combinations. Given its rapid effect on viral suppression, there is increasing use of the RAL, particularly in women who initiate therapy late during pregnancy. Dolutegravir (DTG) was not yet available at the time this study was initiated. Alternative backbone and base are AZT/3TC and LPV/r (DHHS, 2016). Canadian guidelines similarly recommend two NRTIs plus a PI/r as the preferred base due to its safety and efficacy in pregnancy (Money *et al.*, 2014). While US guidelines reserve EFV-based regimens for women who are on medications with significant interactions with PIs (DHHS, 2016) However, since 2013, EFV-containing regimens (usually with TDF/FTC), are widely recommended as first-line regimens for both HIV-infected adults and pregnant women (WHO, 2013).

With this in mind, I chose to study the following cART regimens: TDF/3TC/EFV, widely used in pregnancy in resource-limited settings; AZT/3TC/LPVr, recommended and among the most frequently used cART during pregnancy, including in North America (Money *et al*., 2014; DHHS, 2016); and ABC/3TC ± LPV/r, EFV, or RAL. The latter selection allowed us to evaluate and compare cART regimens sharing a commonly used backbone in combination with a base belonging to different drug classes, including an InSTI, as use of this class of ARV in pregnancy is expected to increase.

The negative control was the drug vehicle, 0.1% DMSO. I ascertained that 0.3% DMSO, the vehicle for EFV and LPV at 20× C_{max}, exerted no effect on cell viability and mtDNA content (data not shown). Positive controls included d4T (1× C_{max}), and 50 μM H_2O_2. D4T-induced mtDNA depletion is widely demonstrated in model cell lines while H_2O_2 is commonly used to induce oxidative stress and examine its deleterious effects in published cell culture studies. In a

pilot experiment, JEG-3 (Human placental choriocarcinoma) cells in exponential growth (approximately 80% confluent) were exposed to 1000, 100, 10, 1, 0.1, and 0.01 mM H_2O_2 for 2 hours, after which the treated cells were trypsinized, counted and seeded into new vessels. Only cells treated with 0.1 and 0.01 mM H_2O_2 attached and grew following subculturing. Based on these results, I included H_2O_2 at 50 µM as a positive control for non-lethal oxidative stress in this study.

4.2.3 Cell Culture

Human placental choriocarcinoma (JEG-3, ATCC-HTB-36) and T lymphoblast (CCRF-CEM, ATCC-CCL-119) cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, US). All cells were cultured according to ATCC recommendations, and all experiments were performed at 37°C in a humidified atmosphere of 5% CO2/95% air.

For the short-term concentration-dependent experiments, JEG-3 and CEM cells were seeded in triplicate in 6-well plates (200,000 cells per well) and incubated for 6-8 hours, after which the ARVS were added to each well and incubated for 3 days. Cell viability, cell doubling time, and mtDNA content were measured at baseline and day 3 (n=3 technical replicates from independent single experiment). Individual ARVs from four different classes were studied at 1×, 10×, and 20× C_{max}, and these are listed in Table 4.1.

For longitudinal experiments, JEG-3 and CEM cells were seeded as above, then exposed to the selected cART regimens at 1× C_{max}, as well as the three controls, for 21 days. After the treatment phase, the cells were returned to ARV-free medium for ten days, to allow recovery/repair and mimic cART interruption. Every three days, cells in two wells were separately harvested, and cells from the third well were subcultured, counted, and seeded into

triplicate wells for the next time point of the experiment. Of note, ARVs were added to each well following 6 hours incubation after subculturing at each time point of treatment phase.

For subculturing of JEG-3 cells, media was removed, and each well was first rinsed with 200 µL 0.25% trypsin, 0.03% EDTA solution (Life Technologies, Eugene, OR, US), then exposed to 400 µL of trypsin EDTA solution for 5 min at 37°C (until the cells detached). Fresh medium (600 µL) was then added to each well. These cell suspensions were used for cell counting and seeding (200,000 cells/well) for next time point. To harvest the JEG-3 cells, media was removed, and 400 µL of AL buffer was added to each well. The plates were incubated at room temperature for 5 min (until all cells were detached and lysed), and 200 µL of this cell suspension was collected for extraction.

For subculturing of CEM cells, 3 mL of cell culture well were collected from, each well and centrifuged at 125 x g for 10 min. The supernatant was removed, and the cells resuspended in 1 mL of fresh medium. These cell suspensions were used for cell counting and seeding the wells (200,000 cells/well) for next time point. For harvesting of CEM cells, 1.5 mL from each well was centrifuged as above and the cell pellet resuspended in 200 µL of AL buffer. These protocols allowed us to extract DNA extraction from approximately 1.5 million cells or less, to prevent exceeding the extraction column's capacity.

Cell viability (trypan blue exclusion) and cell doubling time were measured at each passage, using a haemocytometer (Hausser Scientific, Horsham, PA, US) and automated cell counter (Bio-Rad, Mississauga, ON, Canada) for JEG-3 and CEM cells, respectively. MtDNA content was measured at baseline (6-8 hours after seeding), days 3, 9, 12, and 21 during the treatment phase, and days 24 and 30 of the recovery phase. The longitudinal experiments were performed three times independently.

4.2.4 MMqPCR mtDNA Content

For both cell lines, DNA was extracted using QIAmp DNA mini kit (Qiagen, Toronto, ON, Canada) on the QIAcube (Qiagen, Toronto, ON, Canada) according to the manufacturer's "Blood and body fluid" protocol with the modification that, given that the cell culture samples were already in AL buffer, the latter was replaced with PBS.

For the short-term concentration-dependent experiments, mtDNA content was measured by a monoplex real-time qPCR assay adapted from Côté *et al.*(2002). Briefly, the qPCR conditions were as previously described (Côté *et al.*, 2002), but contained no probes, and the reaction was carried out with the SYBR Green Master (Roche, Basel, Switzerland). A standard curve was prepared with serially diluted plasmid DNA containing the two genes of interest. Both genes were quantified separately in duplicate on the same 96 well plate along with two internal controls, for a total of 20 samples per run. Results are expressed as the relative ratio between mtDNA and nDNA, according to the standard curve. The intra- and inter-run coefficients of variation were 5% and 10%, respectively.

For the longitudinal experiments, mtDNA content was determined using a novel monochromatic multiplex qPCR (MMqPCR) assay recently developed by Anthony Hsieh, another student in our group (Hsieh *et al*., manuscript submitted), also on a LightCycler 480. The latter allows the quantification of both the mitochondrial and nuclear genes, the non-coding mitochondrial D-loop region and albumin, respectively, in the same well. The correlation between the monoplex real-time qPCR and monochromatic multiplex qPCR (MMqPCR) assays is presented in Figure 4.1. Once again, mtDNA content was expressed as mtDNA/nuclear DNA ratio (mtDNA/nDNA). The primer sequences are presented in Table 4. 2. For each 10 µl reaction, 2 µl of sample DNA was added to 8 µl of the master mix containing 1x FastStart SYBR

Green Master (Roche, Basel, Switzerland), 1.2 mM EDTA, and each of the four primers at 0.9 μM. The primers were purified via high-performance liquid chromatography (Integrated DNA Technologies, Coralville, IA, USA). On each 96-well LightCycler 480® Multiwell plate (Roche, Basel, Switzerland), a standard curve of 7 standards, a negative control, and two internal controls (ICs) were included. This allowed quantification of 40 samples in duplicate per plate. The thermal cycling profile of the assay is presented in Table 4.3. All qPCR assays were done on the LightCycler® 480 platform (Roche Basel, Switzerland), with software version 1.5.1.62 SP2. The standard curve was generated by serial dilution (1:5) of two cloned plasmid DNA containing the albumin and D-loop regions of interest mixed in a 1:50 ratio. The range of standard curve was between 5,075,625 - 325 copies of albumin, and between 237,952,683 -15,229 copies of D-loop, resulting in a 15,625-fold linear range (R^2>0.99). The two ICs were derived from a 1:4 dilution of pooled volunteer WB DNA (low mtDNA content), and an extract from cultured SKBR3 cells (Manassas, VA, US) (high mtDNA content). The negative control contained elution buffer (buffer AE) (Qiagen, Toronto, ON, Canada) in place of template DNA.

Because the LightCycler 480 instrument software does not currently permit dual signal acquisition processing, data were exported in text format then imported into Microsoft Excel to separate 74°C (D-loop) acquisitions and 88°C (albumin) acquisitions data by sorting the program and segment markers in the LightCycler 480 software output. The 74°C acquisition was defined as program three segment three, and the 88°C acquisition was program three segment five. Acquisition-delineated data were then converted to grid format in LC480 Conversion version 2.0, a free software which is accessible from the Heart Failure Research Center in Amsterdam, Netherlands, and available online (Heart Failure Research Center, http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LC480Conversion.zip&descrip

tion=LC480Conversion:%20conversion%20of%20raw%20data%20from%20LC480&sub=LC480Conversion, last accessed December 21, 2015). Baseline corrections and C_T calculations were obtained using LinRegPCR version 2012.1 (Heart Failure Research Center). The software establishes a baseline and aligns the exponential phase of the fluorescence curve for all samples to produce a common window of linearity. The C_T is then defined as one cycle below the upper bound of the window of linearity to ensure that the fluorescence threshold is reached during exponential amplification. Individual sample efficiencies generated by LinRegPCR were not used here but rather, the PCR efficiency for each acquisition was calculated from the standard curve included on each plate.

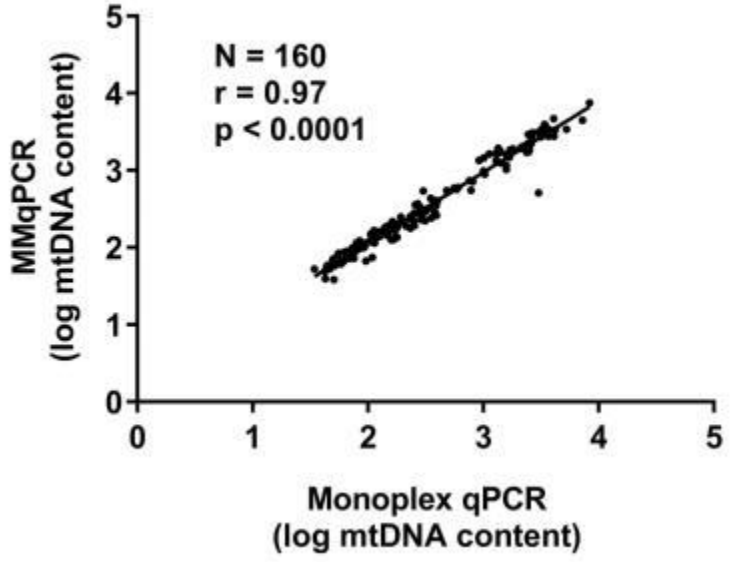

Figure 4.1. Pearson's correlation between log mtDNA content determined by monoplex real-time quantitative PCR (qPCR) and monochromatic multiplex qPCR (MMqPCR). N=160, Pearson's r is shown (Some data provided by Anthony Hsieh, Côté Lab).

Table 4.2. Forward and reverse primer sequences used to measure mtDNA content using monochrome multiplex qPCR assay.

	Primer identifier	Nucleotide Sequence*
Albumin	Albu F	5'cggcggcgggcggcgcgggcTGGGCGGAAATGCTGCACAGAATCCTTG-3'
	Albd R	5'-gcccggcccgccgcgcccgTCCCGCCGGAAAAGCATGGTCGCCTGTT-3'
D-loop	D-loop F	5'-ACGCTCGACACACAGCACTTAAACACATCTCTGC-3'
	D-loop R	5'-GCTCAGGTCATACAGTATGGGAGTGRGAGGGRAAAA-3'

*Upper-case nucleotides represent genome complimentary regions, and lower-case nucleotides represent non-complimentary GC-clamps. Albumin, Alb

Table 4.3. Thermal cycler program for the monochrome multiplex qPCR assay.

Program	No. of cycles	Target temp (°C)	Acquisition mode	Hold time (mm:ss)	Temp. ramp rate (°C/s)
Pre-incubation	1	95	None	15:00	4.4
Amplification	40	94	None	00:15	2.2
		62	None	00:10	2.2
		74	Single	00:15	4.4
		84	None	00:10	4.4
		88	Single	00:15	4.4
Melting Curves	1	95	None	01:00	4.4
		45	None	00:01	2.0
		95	Continuous	---	---
Cooling	1	40	None	00:01	1.5

4.2.5 Relative TL Measurement

For the longitudinal experiments in JEG-3 cells, TL was measured using the MMqPCR assay described in Chapter 2.

4.2.6 Fluorescent Microscopy- Mitochondrial Morphology

4.2.6.1 Cell culture

Live frozen cells, harvested at day 21 and 30 of the longitudinal experiments, suspended 10% DMSO and stored in liquid nitrogen vapour were thawed. They were then cultured again, in the same condition as at the time of harvesting. Prior to seeding, glass coverslips (Sigma-Aldrich, Oakville, ON, Canada) were inserted into each well of 12-well plates, and JEG-3 cells were seeded at 50,000 cells per well.

4.2.6.2 Transfection

To visualize mitochondria, JEG-3 cells were transfected with mitochondrially targeted enhanced yellow fluorescent protein DNA construct (mt-eYFP), generously provided to Dr. Rintoul by Dr. Roger Y. Tsien (University of California, San Diego, US). Mt-eYFP contains the targeting sequence for complex IV of the electron transport chain, cytochrome c oxidase, which allows visualization of the mitochondrial matrix using the mammalian expression vector pCDNA3 (Llopis *et al*., 1998). Transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, US) as described below. Briefly, for each well, 0.5 µg of mt-eYFP and 1.0 µL of Lipofectamine 2000 were diluted in two separate tubes containing 50 µL of serum-free medium for JEG-3 cells. After incubation at room temperature for 5 min, the mt-eYFP solution was mixed with the Lipofectamine 2000 solution and the incubation continued for 20 min. During this time, the volume of media in each well of 12 well plates was adjusted to 400 µL. Then, 100 µL of the transfection mixture was applied to each coverslip and incubated

for 5-6 h at 37°C. Transfection was terminated by replacement of the transfection mixture with 1.0 mL of fresh JEG-3 medium. JEG-3 cells were incubated for a minimum of 12-16 h to allow for transgene expression before fluorescence microscopy

4.2.6.3 Fluorescence Imagining- Mitochondria Morphology

Live cell imaging, and data acquisition were performed using HC Image 4.3 (Hamamatsu Corp., Bridgewater, NJ, US) on a Nikon EclipseTE2000E inverted fluorescence microscope and a Lambda-LS XenonArc lamp light source. Images were captured with a Hamamatsu ORCA-ER camera. All images were taken with a 40×0.6 Numerical Aperture (PlanFluor ELWD) objective lens with a binning of 1 at ambient temperature. Each coverslip was imaged in a magnetic microincubation chamber (Quorum Technologies, Guelph, ON, Canada) in 500 µl of HEPES Buffered Saline Solution (HBSS) pH 7.4.

4.2.7 Analysis and Presentation of Data

For the short-term concentration-dependent experiments, all values presented are the mean ± SD of 3 technical replicates normalized to control values (*i.e.* cells treated with 0.1% DMSO). Longitudinal experiments were performed three times independently. Values for cell viability and cell doubling time are obtained from one technical replicate in each experiment, while values for mtDNA content were obtained from the average of two technical replicates in each experiment. All the longitudinal results were normalized to those of the control well (0.1% DMSO) at each time point of the experiment and expressed as mean ± 1 SD (n=3 independent replicates). All graphs were prepared using GraphPad Prism v.3.02 software (La Jolla, CA, USA). For mitochondria morphology, representative images are shown for each condition.

4.3 Results

4.3.1 Short-term Concentration-dependent Experiments

Both cell lines showed similar responses to ARVs. After three days of exposure, no noticeable differences in cellular proliferation, as indicated by either doubling time (Figure 4.2), or viability were observed in the cells treated with AZT and 3TC, irrespective of treatment concentration. At 10× and 20× C_{max}, a moderate effect was detected in cells treated with d4T, FTC, and TDF. ABC at 1× and 10× C_{max} did not alter the cellular proliferation in either cell types, but it did so at the highest concentration in CEM cells. Among examined NNRTIs, EFV treatment at 10× and 20× C_{max} provoked a substantial concentration-dependent decrease in the cellular proliferation and viability. This effect was most pronounced in the JEG-3 cells, where exposure to EFV at 20× C_{max} more than doubled the cell doubling time in addition to decreasing viability to 38%. Of note, JEG-3 cells exposed to NFV at 20× C_{max} exhibited a substantial decrease in doubling time, but this effect was not seen with CEM cells. LPV at and 10× and 20× C_{max} decreased cellular proliferation in either cell types while for ATV, RTV and RAL, only the 20× C_{max} treatments showed marginal effect.

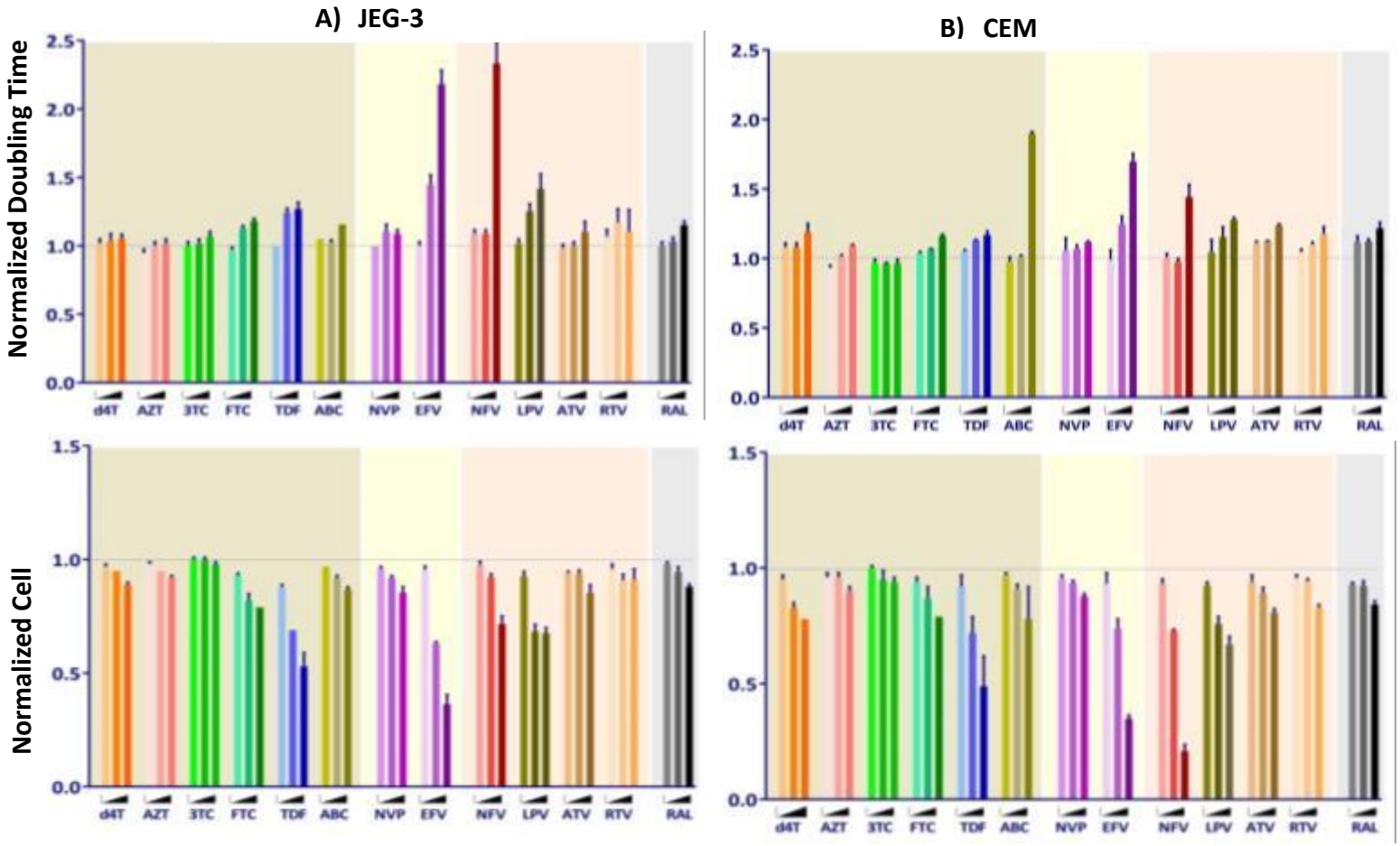

Figure 4.2. Concentration-dependent effect of individual ARVs on JEG-3 and CEM cell viability and doubling time. JEG-3 (A) and CEM (B) cells were exposed to 1, 10, and 20× C_{max} concentration of each ARV for 3 days. ARVs included NRTIs: d4T, AZT, 3TC, FTC, TDF, ABC; NNRTIs: NVP, EFV; PIs: NFV, LPV, ATV, RTV; InSTI: RAL. Data (mean ± 1 SD, n=3 technical replicates from a single independent experiment) were normalized to control values (cells treated with 0.1% DMSO, represented by the horizontal line at 1).

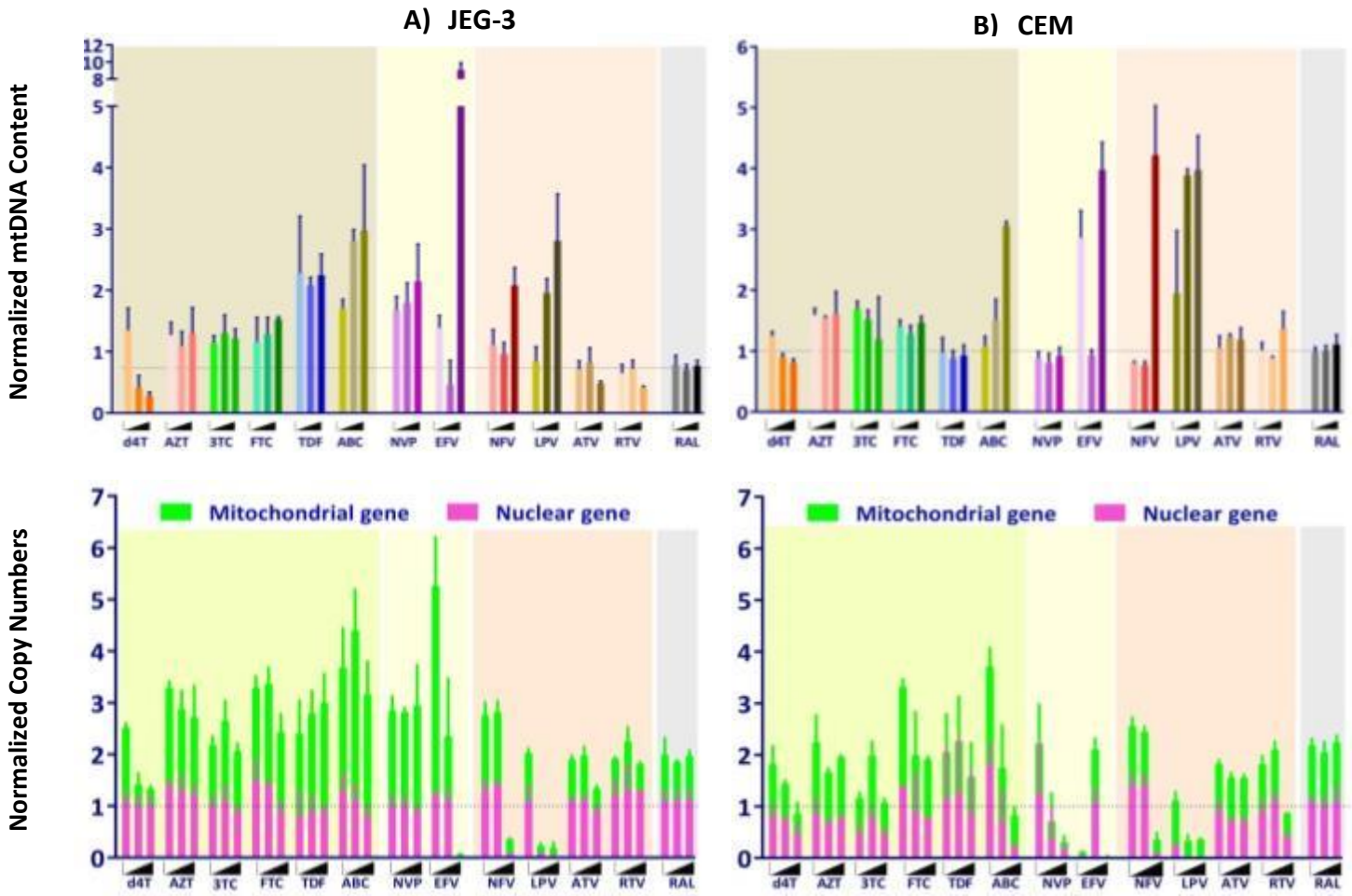

Figure 4.3. Concentration-dependent effect of individual ARVs on JEG-3 and CEM cell mtDNA content. JEG-3 (A) and CEM (B) were treated with 1, 10, and 20× C_{max} ARV for 3 days, after which mtDNA content and the effect on the mitochondrial gene copy number (green) and nuclear gene copy number (pink) were determined. ARVs included NRTIs: d4T, AZT, 3TC, FTC, TDF, ABC; NNRTIs: NVP, EFV; PIs: NFV, LPV, ATV, RTV; InSTI: RAL. Data are presented as mean ± 1 SD, (n=3 technical replicates from a single independent experiment), normalized to control cells treated with 0.1% DMSO, and represented by the horizontal line at 1.

Among all NRTIs tested, only d4T resulted in mtDNA depletion, and this effect was only observed at the higher concentrations. At 1× C_{max} d4T exposure led to a slight ~20% increase in mtDNA content. For all other NRTIs, an increase in mtDNA content was exhibited in both cell types, an effect that was more pronounced in JEG-3 cells treated with TDF or ABC although the latter also increased mtDNA in a concentration-dependent manner in CEM cells. Among all ARVs tested, EFV exerted the largest cytotoxic effect in both JEG-3 and CEM cells, undermining their proliferation and viability at 10× and 20× C_{max}. These effects were concurrent with a mixed effect seen in the mtDNA content, where mtDNA increased at 1× and 20× C_{max} but was unchanged or slightly decreased at 10× C_{max}. As revealed in Figure 4.3, mtDNA content changes in JEG-3 cells were primarily due to changes in mtDNA copy number per cell as the nDNA copy number for each well remained fairly constant. The only conditions that affected nDNA downward were EFV and NFV 20× C_{max} as well as the two higher LPV concentrations, and these treatments also led to cell death. For CEM cells (Figure 4.2), the effects were similar to those observed with JEG-3 cells, but the nDNA copy numbers were lower, indicative of cell death, particularly for ABC, NVP, EFV, NFV, and LPV. RAL is the only InSTI tested in this study, and it did not exert any noticeable changes in mtDNA content in either cell line.

4.3.2 Longitudinal Experiments

4.3.2.1 Cell Viability, Doubling Time, and mtDNA Content

The longitudinal effects of 1× C_{max} cART regimens and positive controls (d4T and H_2O_2) on cell viability and mtDNA content in JEG-3 cells are shown in Figure 4.4. JEG-3 cells treated with 1× C_{max} d4T exhibited a notable drop in mtDNA content that reached its nadir at day 9 of treatment and remained low throughout the treatment phase. After removing the pressure of d4T, mtDNA content rebounded by day 24 and by day 30, reached a level above baseline. As seen in

the concentration-dependent experiments, despite marked effects on mtDNA content, d4T treatment did not alter cell viability and doubling time (Figure 4.2). H_2O_2 induced an approximately 25% decrease in cell viability and mtDNA content after three days. However, mtDNA content returned to baseline level by day 9 and remained stable throughout the experiment.

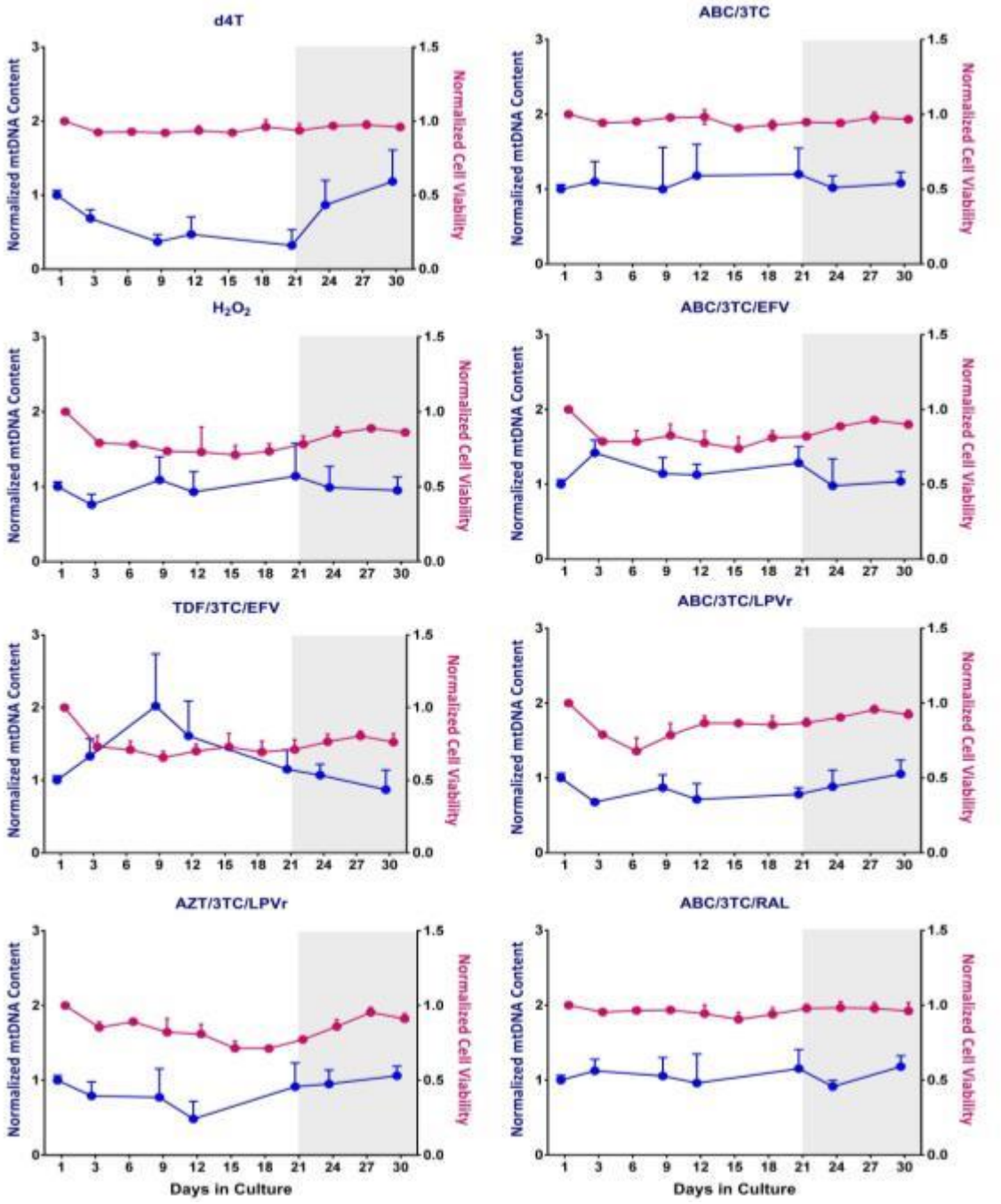

Figure 4.4. Longitudinal effect of 1×C_{max} cART regimens on JEG-3 cells. Treatments included, TDF/3TC/EFV, AZT/3TC/LPVr, ABC/3TC/EFV, ABC/3TC/LPVr, ABC/3TC/RAL, and ABC/3TC backbone, d4T (control for mtDNA depletion) all at 1× C_{max}, and H_2O_2 at 50µM (control for oxidative stress) on mtDNA content (left axis, blue) and cell viability (right axis, red). Cells were cultured in presence of each treatment for 21 days (treatment phase, white background) and then were returned to ARV-free medium for 10 days, to allow recovery (recovery /repair phase, grey background). Values for cell viability obtained from a single well while those for mtDNA content were obtained from n=2 technical replicates in each experiment. All results are normalized to control values (cells treated with

0.1% DMSO, represented at 1) at each time point of the experiment, and expressed as mean ±1 SD (n=3 independent experiments).

Among all cART regimens tested, TDF/3TC/EFV provoked the highest increase (1.5-2.0 folds) in mtDNA content, which was most pronounced on days 9 and 12, then gradually decreased toward baseline level. Although error bars were large on day 9, Figure 4.8 indicates that all three replicates showed a similar pattern. The observed variability was amplified by subtle differences in the timing and amplitude of the change in mtDNA content. Likewise, mtDNA content in JEG-3 cells increased following exposure to either TDF or EFV at 1× C_{max} in the short-term concentration-dependent experiments. These effects on mtDNA content were concurrent with a 30% decrease in cell viability starting after three days of treatment and persisting throughout the treatment phase. Another EFV-based treatment tested in this study, ABC/3TC/EFV, provoked approximately half a fold increase in mtDNA content, accompanied with lower cell viability by 20-30%. In contrast with TDF/3TC/EFV treatment, AZT/3TC/LPVr exerted up to a half fold decrease in the mtDNA content, and lowered viability by 15-30%. JEG-3 cells showed similar trend in response to both LPVr-based treatments in this experiment. JEG-3 cells treated with ABC/3TC/LPVr exhibited a 30% decrease in mtDNA content and cell viability (15-35%). Minimal changes were seen in cells treated with ABC/3TC backbone and ABC/3TC/RAL. Of note, the viability and mtDNA content changes seen during the treatment phase were either partially or completely reversed during the recovery phase.

The longitudinal effect of 1× C_{max} cART regimens and positive controls (d4T and H_2O_2) on cell viability and mtDNA content in CEM cells is shown in Figure 4.3. As expected, mtDNA content decreased in CEM cells treated with d4T. In a pattern similar to JEG-3 cells, mtDNA content rebounded by day 24 and substantially increased by day 30 but unlike JEG-3 cells, some

cytotoxicity was observed with CEM cells, as reflected by lower cell viability. H_2O_2 treatment provoked a three-fold increase in mtDNA content, along with a negative effect on the cell viability (~30%).

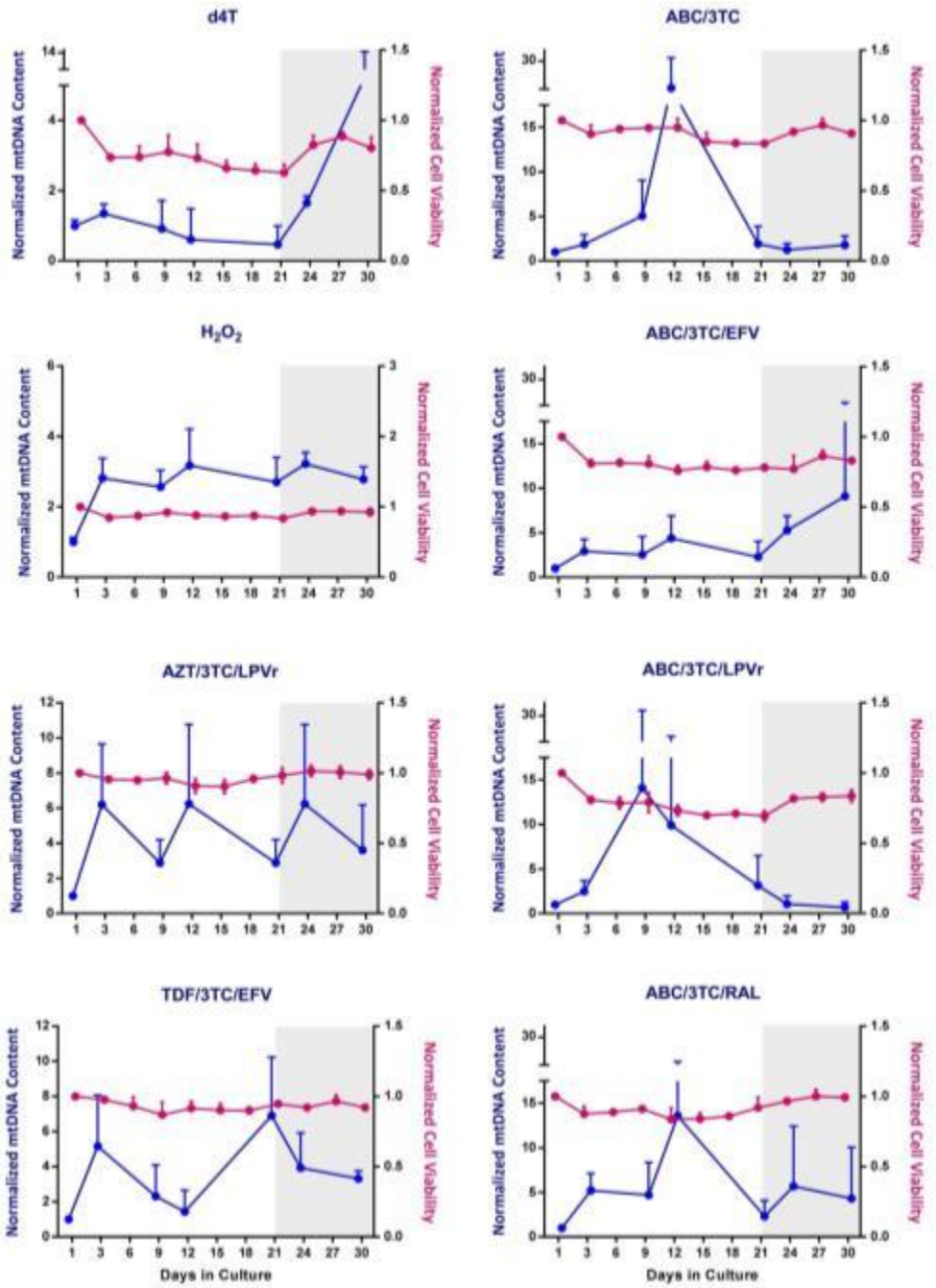

Figure 4.5. Longitudinal effect of 1×C_{max} cART regimens on CEM cells. Treatments included TDF/3TC/EFV, AZT/3TC/LPVr, ABC/3TC/EFV, ABC/3TC/LPVr, ABC/3TC/RAL, and ABC/3TC backbone, d4T (control for mtDNA depletion) all at 1× C_{max}, and H_2O_2 at 50µM (control for oxidative stress) on mtDNA content (left axis, blue) and cell viability (right axis, red). Cells were cultured in presence of each treatment for 21 days (treatment phase, white background) and then were returned to ARV-free medium for 10 days, to allow recovery (recovery /repair phase, grey background). Values for cell viability obtained from a single well while those for mtDNA content were obtained from n=2 technical replicates in each experiment. All results are normalized to control values (cells treated with

0.1% DMSO, represented at 1) at each time point of the experiment, and expressed as mean ± SD (n=3 independent experiments).

In general, changes in mtDNA content and variability were more pronounced for CEM cells than JEG-3 cells. CEM cells exposed to ABC/3TC/ EFV treatment exhibited an overall increase in mtDNA content. However, a mixed effect was seen with TDF/3TC/EFV whereby mtDNA appeared to increase at days three and 21 but was lower at day 9 and 12 of the treatment phase. These effects were followed by a rapid decline early in the recovery phase. Unlike JEG-3 cells, CEM cells exposed to LPVr-based regimens, including AZT/3TC/LPVr and ABC/3TC/LPVr showed increased mtDNA content, which was partially reversed in the recovery phase. This increase in mtDNA content in CEM cells is consistent with the increase in CEM mtDNA content following treated with LPV at 1× C_{max} in the concentration-dependent experiment, something that was not seen with JEG-3 cells (Figure 4.2). Also, unlike JEG-3 cells, all ABC/3TC containing regimens substantially (5-10 fold) increased mtDNA content in CEM cells. These effects were concurrent with reduced cell viability, particularly for EFV and LPVr-based cARTs, but as with JEG-3 cells, the negative effects on cell viability were either partially or completely reversed during the recovery phase. The error bars for several time points are large but as seen for CEM cells treated with ABC/3TC (Figure 4.6), the same pattern is observed in each independent experiment, but at different intensity, and slightly different timing. The same pattern was seen for other treatments with large variability (data not shown). My data therefore suggest that different population of cells respond to treatment similarly but at different rate and potentially with some lag with respect to time points of experiment.

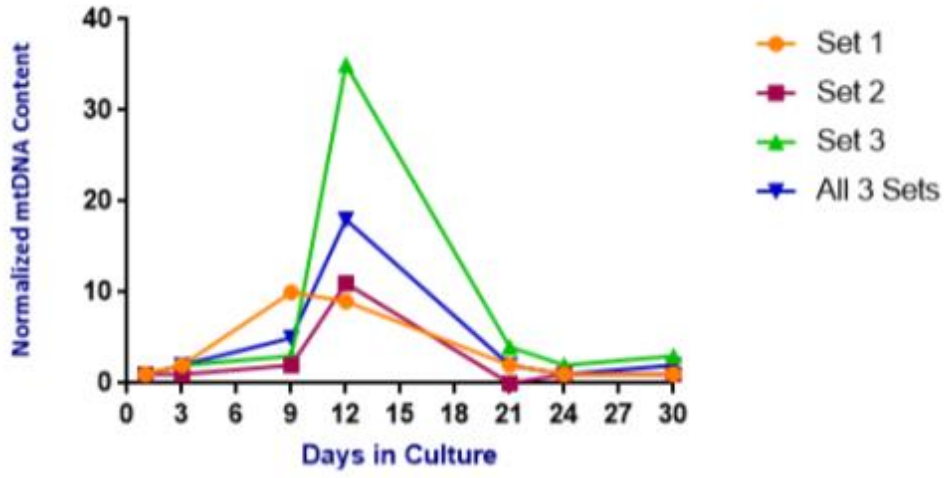

Figure 4.6. Longitudinal effect of 1×C_{max} ABC/3TC on mtDNA content in CEM cells. Values for each set (one independent experiment) were obtained from n=2 technical replicates in each experiment. All results are normalized to control values (cells treated with 0.1% DMSO).

4.3.2.2 Relative Telomere Length

Telomere length was measured in the same DNA extracts used for mtDNA measures. In general, TL remained stable throughout the 21 days of exposure, except for H_2O_2, TDF/FTC/EFV, and AZT/3TC/LPVr treatments. All three induced a large apparent increase in TL (Figure 4.7), where once again, the variability was amplified by differences in intensity and timing of the change in TL (Figure 4.8).

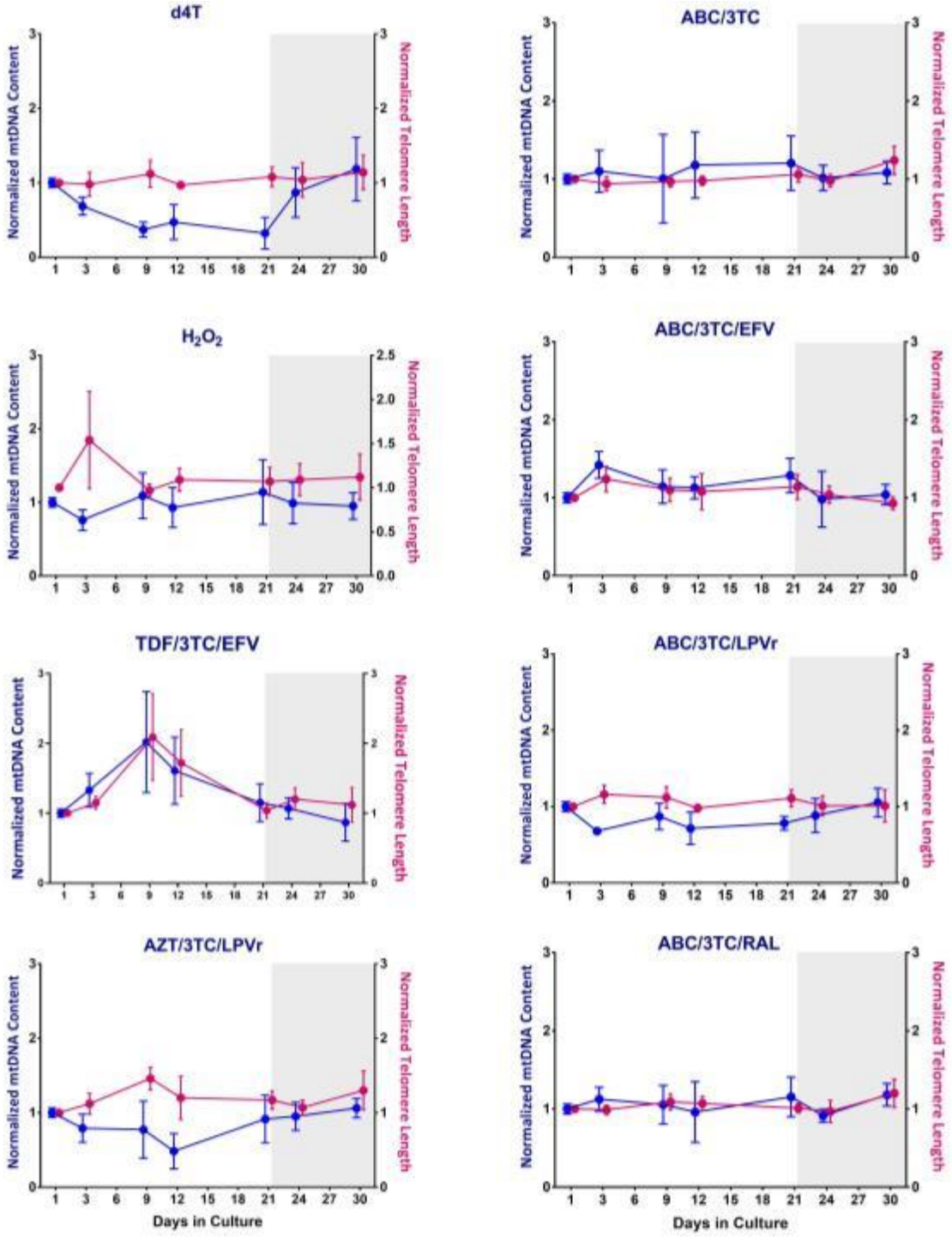

Figure 4.7. Longitudinal effect of 1×C_{max} cART regimens on JEG-3 cells mtDNA and telomere length. Treatments included TDF/3TC/EFV, AZT/3TC/LPVr, ABC/3TC/EFV, ABC/3TC/LPVr, ABC/3TC/RAL, and ABC/3TC backbone, d4T (control for mtDNA depletion) all at 1× C_{max}, and H_2O_2 at 50µM (control for oxidative stress) on mtDNA content (left axis, blue) and Telomere Length (TL) (right axis, red). Cells were cultured in presence of each treatment for 21 days (treatment phase, white

background) and then were returned to ARV-free medium for 10 days, to allow recovery (recovery /repair phase, grey background). All Values obtained from n=2 technical replicates in each experiment. All results are normalized to control values (cells treated with 0.1% DMSO, represented at 1) at each time point of the experiment, and expressed as mean ± 1 SD (n=3 independent experiments).

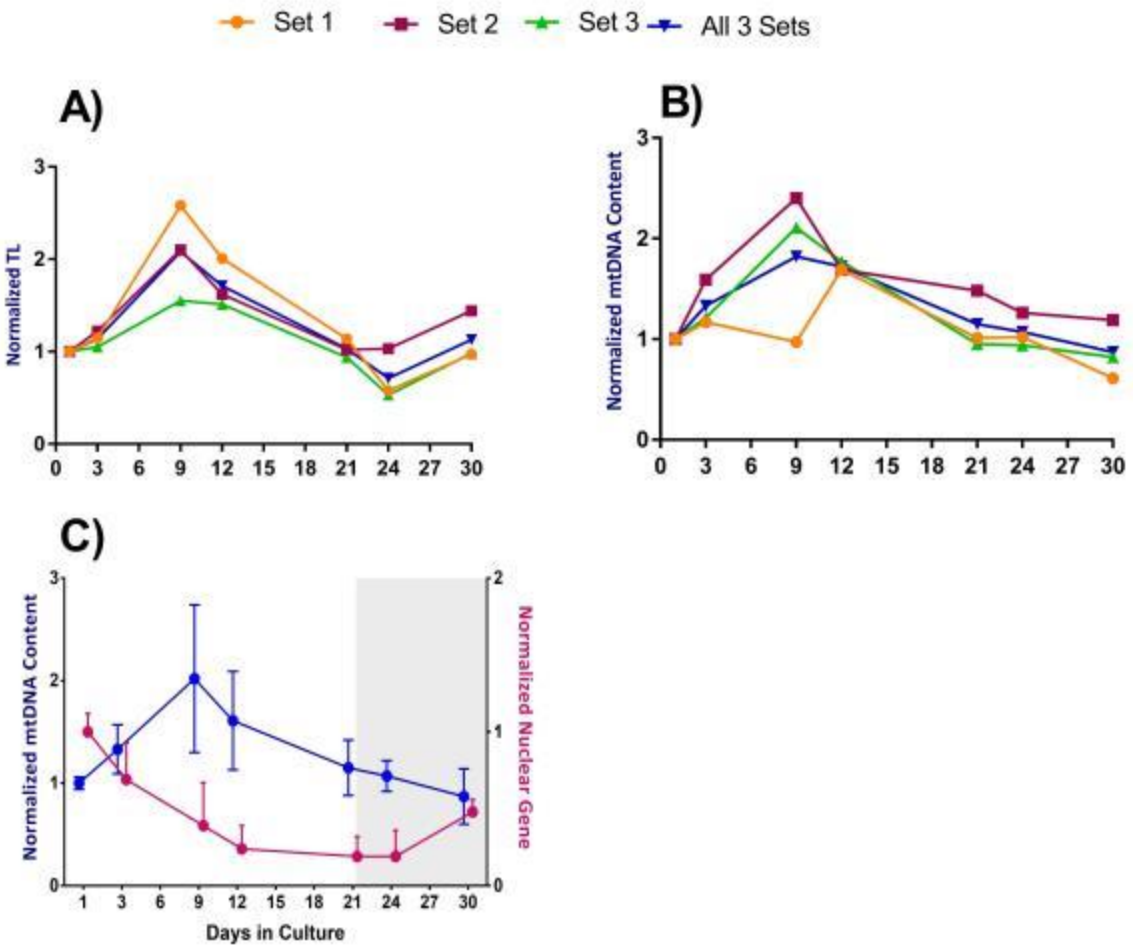

Figure 4.8. Longitudinal effect of 1×C_{max} TDF/3TC/EFV on TL/mtDNA content in JEG-3 cells. Values for each set (one independent experiment) were obtained from n=2 technical replicates in each experiment. All results are normalized to control values (cells treated with 0.1% DMSO)

4.3.2.3 Mitochondrial Morphology

Cells frozen at the end of the treatment phase, on days 21, and at the end of the recovery phase, day 30 of the experiment, were cultured under their initial conditions and transfected in order to visualize the mitochondrial organelles therein. Representative images of mitochondria

morphology are shown in Figure 4.8, for a subset of the longitudinal treatments. Mitochondria in control cells treated with 0.1% DMSO demonstrate diversity in their shape and length, with the majority of them appearing long and filamentous, both on day 21 (A-1), and day 30 (A-2). All mitochondria in cells exposed to 50 µM H_2O_2 at day 21 appear punctate (B-1). After recovery from this treatment, mitochondria appear once again of different length (B-2). At day 21, the density of mitochondria decreased markedly in cells exposed to d4T at $1\times C_{max}$ (C-1), and the majority of the organelles appear of moderate length compared to the long mitochondria observed in panel A. The density of mitochondria increased during the recovery phase from d4T treatment, with more long and mid-length organelles visible (C-2). These results are consistent with the short-term and longitudinal experiments, in which mtDNA content in JEG-3 cells treated with d4T decreased substantially during the treatment phase and recovered once the drug pressure was removed. The majority of mitochondria in cells exposed to ABC/3TC/EFV at $1\times C_{max}$ (D-1) are short in length and close to punctate shape, similar to the organelles seen in panel B after H_2O_2 treatment. After 10 days of recovery from ABC/3TC/EFV treatment, mitochondria appear once again of varying length (D-2). For all treatments, morphological changes were partially reversed during the recovery phase. Of note, another set of immunofluorescence microscopy experiment was performed on a different day. However, the changes in mitochondrial morphology in the JEG-3 cells treated with the remaining ABC/3TC containing treatments and backbone were inconclusive (data not shown).

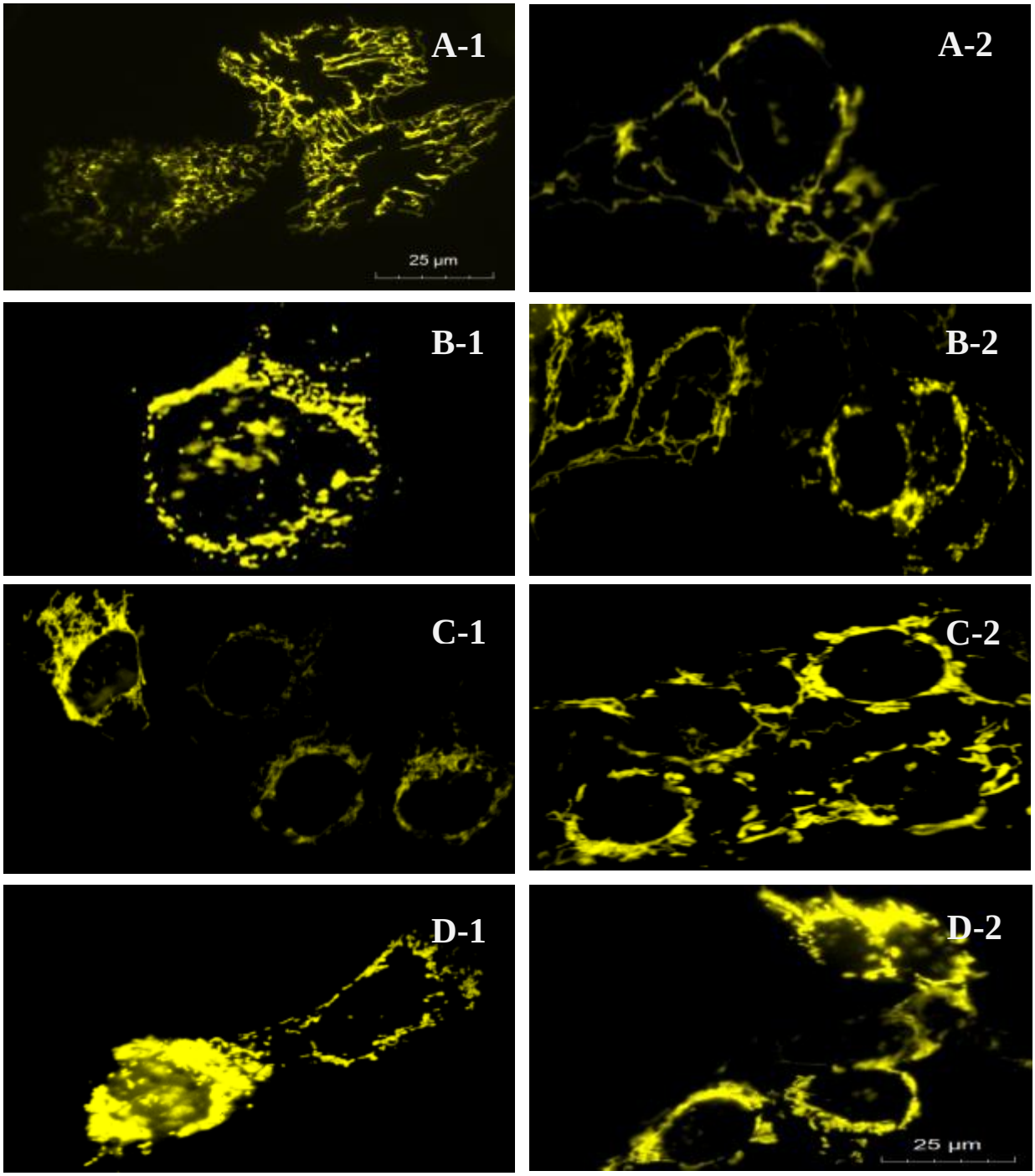

Figure 4.9. Immunofluorescence images of previously harvested JEG-3 cells at day 21 and 30 of the longitudinal experiments, which were cultured again, in the same condition they were in at the time of harvesting. Cells were transfected with mt-eYFP, cells treated with 0.1% DMSO (A), 50µM H_2O_2 (B), d4T at $1\times C_{max}$ (C), and ABC/3TC/EFV at $1\times C_{max}$. The left panels represent cells after 21 days of exposure, while the right panels represent cells on day 30, after 10 days of recovery.

4.4 Discussion

In the era of cART's success, it is crucial to investigate the long-term safety of ARVs at the cellular level, as this consideration may influence cART recommendations for people living with HIV. ARVs can affect cellular aging through a variety of mechanisms, including mitochondrial dysfunction (Capeau, 2011; Deeks, 2011). Much knowledge is acquired investigating clinical samples obtained from HIV-infected cART-treated individuals. However, in clinical studies, it is usually challenging to distinguish possible effects related to HIV infection, versus those related to cART. Given that cART implies three or more ARVs, and the fact that most HIV-infected cART-treated individuals have received multiple regimens over time, reaching conclusions about specific ARVs or regimens is also challenging. In the context of recent guidelines recommending immediate initiation of lifelong therapy without interruption (UNAIDS, 2016), an urgent need for data on the long-term safety of cART is increasingly voiced. In response to this gap in knowledge, I designed *in vitro* experiments to evaluate the mitochondrial toxicity of six cART treatments with extensive use in pregnancy, in two different cell lines, JEG-3 and CEM cells.

Extensive attention has been paid to early generation NRTIs-induced acute mitochondrial toxicity through inhibition of POLγ leading to mtDNA depletion (Lim and Copeland, 2001; Côté *et al.*, 2002) and mutations (Kakuda, 2000). However, data remain scarce for newer NRTIs and other ARV drug classes, as well as potential interactions between different ARVs when used in combination. In this study, apart from expected mtDNA depletion with d4T, most other NRTIs showed either little effect or led to increase mtDNA content in both cell lines.

In agreement with other studies in HepG2 cells (Walker *et al.*, 2002), I detected an additive or synergistic long-term cytotoxicity for ABC and 3TC used in combination (Venhoff *et*

al., 2007), despite a lack of cytotoxicity with each of these ARVs individually (Walker *et al.*, 2002; Venhoff *et al*., 2007). ABC/3TC reduced cell viability, and this was accompanied by an increase in mtDNA content, which was more pronounced in CEM cells. Cell type or tissue-specific differences with respect to NRTIs toxicity could be explained by differences in pro-drug activation and elimination and/or mitochondrial uptake pathways (Lewis and Dalakas, 1995; Brinkman *et al*., 1998; Kakuda, 2000; Becher *et al*., 2003; Anderson *et al.*, 2004; Borroto-Esoda *et al*., 2006; Sun *et al.*, 2014). T-lymphoblastoid cells have been widely used to investigate NRTI-induced mitochondrial toxicity and efficiently activate NRTIs (Chen *et al.*, 1991; Medina *et al*., 1994).

EFV is the most widely used NNRTI. Previous studies in cultured hepatocytes showed that it can affect mitochondria directly and indirectly (Apostolova *et al*., 2010), and that low to moderate concentrations (1 and 2.5× C_{max}) can increase mitochondrial mass, alter mitochondrial morphology, and trigger autophagic flux, hence mitophagy, likely a cell survival mechanism. At higher concentrations (5×C_{max}) however, EFV was reported to block autophagic flux and induce apoptosis (Apostolova *et al*., 2011). In line with this, I observed normal cell viability in both JEG-3 and CEM cells treated with 1×C_{max} EFV but higher concentrations provoked cell death accompanied with large changes in mtDNA content. Indeed, EFV-based treatments (TDF/FTC or ABC/3TC) increased mtDNA content, with more pronounced effect in CEM cells, with the ABC/3TC/EFV treatment giving rise to punctate-shaped mitochondria in JEG-3 cells, suggestive of compensatory autophagy/mitophagy to preserve mitochondrial health, as reported in hepatic cells treated with EFV (Apostolova *et al*., 2011a). My data suggest an upregulation of mitochondria biogenesis in response to ARV-induced cellular stresses and/or mitochondrial damage, resulting in the observed increased mtDNA content. I therefore postulate that an

increase in mtDNA content is a marker for a different type of toxicity than the mtDNA depletion induced by some NRTIs and is not necessarily indicative of lower toxicity. This effect on mtDNA content may have confounded early studies aimed at evaluating ARV mitochondrial toxicity based on blood cell mtDNA content (Montaner *et al.*, 2004). In addition to NRTI-mediated effects, PIs can increase ROS production and oxidative stress in cell culture models (Apostolova *et al.*, year; Blas-García *et al.*, 2011), triggering premature senescence *in vitro* (Caron *et al*. 2008). In the current study, JEG-3 and CEM cells responded differently to LPV/r-based treatments. Different PI cytotoxic effects in different cell types has been observed before (Zhong *et al*., 2002; Vidal *et al*., 2006). Importantly, my findings with respect to the bidirectional effects of different ARVs and the cell-specific alterations in mtDNA content in response to one cART treatments highlight the importance of investigating ARVs alone and in combinations, and in different cell types in order to assess their possible effects on the human body or the developing fetus.

Recent studies showed no evidence of mitochondrial toxicity was observed in primary rat neurons and hepatocytes treated with RAL (Blas-García *et al*., 2014), in agreement with existing clinical experience of rare cases of hepatic adverse events with RAL (Capetti *et al*., 2012). However, data remain scarce with respect to *in vitro* and *in vivo* toxicity of relatively newer ARVs, such as RAL. I showed an increase in mtDNA content in CEM cells treated with a RAL containing cART regimen, but minimal changes in JEG-3 cells, suggesting cell-specific effects.

Overall, most cART regimens studied herein induced increased mtDNA content, postulated to reflect mitochondria biogenesis in response to stresses, including oxidative stress (Lee and Wei, 2005). A cell's mtDNA is a mixture of damaged and functional mtDNA

fragments, hence mtDNA content was proposed to be a marker of mitochondrial dysfunction (Malik and Czajka, 2013).

In my longitudinal study, alterations in mtDNA content were mostly reversible once the drug pressure was removed, reinforcing the notion that these changes were in response treatment-induced stress, but also suggesting that tissues can adapt. Accumulation of mutated mtDNA, which some have termed "mitochondrial aging" (Payne *et al*, 2011) is suggested to arise primarily from the clonal expansion of pre-existing mtDNA mutations (Payne *et al*., 2011). In turn, the majority of these somatic mutations (in the general population) are believed to arise from POLγ errors. In the context of HIV/ART however, pre-existing mtDNA mutation could be further amplified by chronic inflammation and/or compromised POLγ fidelity in the presence of nucleotide imbalance in the mitochondria (Payne *et al*., 2011). In light of this, my findings of increased mtDNA content suggest accelerated mtDNA turnover and/or mitochondrial proliferation following exposure to cART treatments. Both could favor clonal expansion of mtDNA mutations, a phenomenon that merits further investigation.

The apparent increase in JEG-3 relative TL in the presence of both EFV- and LPV/r-based cART regimens is likely secondary to cell adaptation and/or cell death favoring the survival of cells with longer TL. Concurrently, exposure to EFV-based cART led to higher mtDNA while LPV/r-based cART resulted in lower mtDNA, indicating that different effects are at play. This finding needs further investigation considering the possible links between mitochondrial damage and telomere shortening theories of aging (Sahin and Depinho 2010; Sahin *et al*. 2011)

4.5 Strength and Limitations

The major strength of this study was the longitudinal design, which allowed better insight into mtDNA content and its dynamics, and experimentally addressing whether changes induced by cART exposure are reversed upon drug pressure removal. Furthermore, I evaluated six cART regimens relevant to pregnancy, a context where historically, women initiated and stopped cART with each pregnancy. This study also has several limitations. It was conducted in transformed cell lines, which likely have different cellular bioenergetics than primary or stem cells. Mitochondrial toxicity in patients often manifests in resting or slowly dividing tissues including muscle, liver, brain, pancreas, and adipocytes following exposure to medium to long-term ART therapy. These tissues are metabolically active and highly dependent on mitochondrial function. Therefore, data generated from short-term experiments using highly proliferating and poorly differentiated cells may or may not adequately translate to humans. However, the month-long experiments in two independent cell models partially address these issues, and represent the longest study to date. The major limitation of this study is the high variability in the mtDNA content results, especially in the CEM cells. Therefore, a larger number of independent replicates would be desirable. Furthermore, given that some effects were cell-specific, increasing the number of cell lines (biological replicates) would also strengthen this study.

4.6 Conclusions

In summary, my study suggests that exposure to different ARVs elicits different cellular and mitochondrial responses, involving different mechanisms. This highlights the need to investigate the effect of cART regimens, including newer regimens, especially in the context of pregnancy, given that altered mtDNA may be a marker for cellular metabolism dysregulation, which in turn could interfere with placental function and/or fetal development and growth.

Previous studies by our group and others reported lower maternal LTL (Saberi *et al*., 2017b) and blood mtDNA content in HIV-infected cART-treated pregnant women compared to HIV- women (Poovathingal *et al*., 2012; Money *et al*., 2015). Yet, our group and others also reported that infants born to women living with HIV and treated with cART in pregnancy have higher blood mtDNA compared to infants born to HIV- women (Côté *et al.*, 2008; Ross *et al.*, 2012). Importantly, severe cellular cytotoxicity has not been seen which is highly reassuring for women taking ARV's in pregnancy; however, changes in mtDNA in response to exposure to ARV's requires further study. Taken together, these observations stress the importance of *in vitro* studies to evaluate the cytotoxicity of individual ARVs as well as their interactions in different cell types.

Chapter 5: Conclusions

5.1 Summary of Findings

This research focused on the effects of cART at the cellular level in both clinical samples and cell culture models. The first objective was to measure the dynamics of LTL in HIV-infected cART-treated pregnant women and HIV-uninfected pregnant control women, and the second one was to investigate alterations in mtDNA content and mitochondrial morphology following prolonged exposure to cART regimens that have been extensively used in pregnancy, both in North America and throughout the world.

Longitudinal assessment of LTL demonstrated that LTL was relatively stable throughout pregnancy in both HIV-infected and HIV-uninfected women. I found a statistical interaction between maternal age and weeks of gestation, suggesting apparent LTL lengthening over time as pregnancy progresses, in younger women. This may be partially explained by age-related differences in physiological changes during pregnancy, including leukocyte turnover, new leukocyte generation, and hormonal changes between younger and older women.

In spite of a trend suggesting that HIV-infected women who were on cART at visit had longer LTL compared to women who were off cART, this effect was not statistically significant in the longitudinal analysis. These results do not support my initial hypothesis which was cART treatment status would negatively modulate LTL during pregnancy. However, type of cART regimen was showed to be associated with LTL, whereby women treated with a PI/r-based regimen had shorter LTL compared to women who received other regimens, among which un-boosted PI-based regimens were dominant. Smoking throughout pregnancy was the other predictor of shorter LTL among HIV-infected pregnant women. Given that nearly a third of women who smoked throughout pregnancy also self-reported substance use, while none of the

non-smokers did, the negative impact of smoking on LTL may also be also confounded, by other substances of addiction. This extends previous studies reporting the negative effects of environmental factors such as smoking and illicit drugs on LTL (Zanet *et al.,* 2014; Huzen *et al.,* 2014; Müezzinler *et al.,* 2015). It is important to note that the two groups both showed high rates of smoking

Given that the LTL effect size of smoking was similar to my initially hypothesized HIV effect, I examined the reliability of self-reported smoking during pregnancy in our cohorts by measuring plasma cotinine concentration and determining the relationship between the two measures. I found excellent concordance (91%) between plasma cotinine and smoking self-report, indicating that the smoking self-reported data are reliable as a surrogate for tobacco exposure (Saberi *et al.,* 2017b). Furthermore, I re-examined the study groups based on their smoking status and found that women who smoked throughout pregnancy were younger, delivered at an earlier gestational age, were significantly more likely to be HIV+, to have a low income, a history of HCV infection, and report substance use throughout pregnancy.

Consistent with the high rate of smoking in this study, incidence of pre-term birth was markedly higher than national and provincial rates. Given that increased risk of pre-term delivery has also been associated with PI/r regimens (Powis *et al.,* 2011; Sibiude *et al.,* year; Kakkar *et al.,* 2015), the relationship between shorter LTL, pre-term birth, and PI/r requires further investigation.

Much knowledge is learned investigating clinical samples collected from HIV+ cART-treated individuals. However, it is challenging to distinguish between effects related to HIV infection versus those related to cART in the clinical studies. Considering that cART regimens usually contain three or more ARVs and the fact that most HIV+ cART-treated individuals have

received multiple regimens over time, reaching conclusions about specific ARVs or regimens is also challenging. In the context of current guidelines that promote lifelong cART for all persons living with HIV, data on the long-term safety of cART are urgently needed. To address this knowledge gap, I designed *in vitro* experiments to evaluate the mitochondrial toxicity of six cART treatments with extensive use in pregnancy, in two different cell lines, placental (JEG-3) and T-lymphoblast (CEM) cells.

I showed that mtDNA content changes in response to ARV exposure, that this effect can be both bidirectional and cell-specific, and that it appears reversible. Most ARVs and cART studied here induced increased mtDNA content, postulated to reflect mitochondria biogenesis in response to cellular stresses and/or damage, something that could promote the clonal expansion of mtDNA mutations, hence affect cellular aging. I also showed mitochondria morphological changes in JEG-3 cells treated with EFV-based treatment, which is suggestive of increased mitophagy to preserve mitochondrial health. Taken together, I postulated that an increase in mtDNA content is not necessarily indicative of lower toxicity as opposed to mtDNA depletion induced by some early generation NRTIs. Importantly, severe cellular cytotoxicity has not been seen which is highly reassuring for women taking ARV's in pregnancy, however, changes in mtDNA in response to exposure to ARV's requires further study. In summary, my study suggests that exposure to different ARVs elicits different cellular and mitochondrial responses, involving different mechanisms. This highlights the need to investigate the effect of cART regimens in different cell types, particularly in primary or stem cells.

5.2 Significance and Translation of the Study

Given that recent studies suggested possible links between telomeres/telomerase and reproductive health, including fertility and pre-term delivery (Liu *et al.*, 2002; Smeets *et al.*, 2015; Antunes *et al.*, 2015), TL during pregnancy and influential factors should be investigated further. This study provided the first ever reported insight into LTL dynamics during pregnancy and showed that substance use such as smoking exerts large effects.

I showed that cART regimens use in pregnancy, including newer regimens, can alter mtDNA and reflect cellular metabolism dysregulation that could affect fetal development and growth. These results warrant further investigation in both clinical and cell culture studies, including in primary and stem cells, especially with newer ARVs for which less is known.

Taken together, pharmaceutically relevant concentrations of cART regimens are not associated with shorter LTL and cellular cytotoxicity in HIV- infected cART-treated women and cell culture models, which is highly reassuring for HIV-infected women treated with ARV's in pregnancy. Nevertheless, changes in LTL and mtDNA in response to exposure to ARV's requires further study.

5.3 Future Direction

I showed that ARVs can exert bidirectional effects on mtDNA content illustrating the importance of evaluating ARVs alone and in combinations, using multiple mtDNA measures. As such, random somatic mutations on cell culture samples generated as part of this thesis will be quantified using ultra-deep sequencing assay, which is based on an approach first described by Jabara *et al.*(2011). It incorporates a PCR template tag ID in addition to the sample tag. This strategy allows us to differentiate "true" mutations from mutations produced by PCR and sequencing errors. I was involved in the early stages of data analysis of somatic mtDNA

mutations, although the work was primarily done in our lab by Adam Ziada. My initial proposal was to quantify mtDNA mutations in HIV-infected pregnant women treated with cART regimens, as well as in cell culture models following treatment with various cART regimens. As it was required by the mutation assay, blood mtDNA content was measured in 64 HIV-infected and 41 HIV-uninfected women who had biological samples at all three of their pregnancy visits. I also generated the cell culture samples and measured the mtDNA content. My initial plan was to measure mutations on cell culture samples collected after 21 days of exposure to ABC/3TC+ LPVr or EFV or RAL and similarly after ten days of recovery. However, the assay was delayed when Roche no longer supported the 454 platforms, which forced us to move to MiSeq. This in turn affected our bioinformatics pipeline, required new coding, and further validation. However, the issues have now been mostly resolved, and the cell culture samples generated as part of my thesis will undergo ultra-deep sequencing in the near future

Although mtDNA content and somatic mutations enable the assessment of biological or cellular aging, mitochondrial health can also be evaluated by other measures. For example, measuring mitochondrial membrane potential ($\Delta\psi m$) and the presence of reactive oxygen species (ROS) using flow cytometry can provide additional information about the state of mitochondrial health. Another PhD student in our lab, Anthony Hsieh is working on flow-based assessments of mitochondrial health. He established a protocol (or workflow), which uses the cell culture samples for flow cytometry following cell staining with MitoTracker Deep Red, MitoTracker Green, and MitoSOX to measure mitochondrial intermembrane potential ($\Delta\psi m$), mitochondrial mass, and ROS, respectively. Cells with high and low $\Delta\psi m$ are then sorted, and for each sorted population, mtDNA content can be measured using MMqPCR.

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