DYNAMICS OF TELOMERE LENGTH AND MITOCHONDRIAL HEALTH IN RELATION TO COMBINATION ANTIRETROVIRAL THERAPY (cART) EXPOSURE: A COHORT STUDY OF HIV/cART-EXPOSED UNINFECTED CHILDREN AND CELL CULTURE INVESTIGATIONS

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Abstract

Combination antiretroviral therapy (cART) during pregnancy has considerably reduced the risk of mother-to-child HIV transmission and the number of cART-exposed HIV-exposed uninfected (HEU) children is increasing. With current treatment guidelines recommending the initiation of immediate, lifelong cART at HIV diagnosis, women conceive on therapy and HEU *in utero* cART exposure spans the entire gestation period. Many antiretrovirals (ARV) cross the placenta and could exert long-term effects on HEUs. Some ARVs inhibit human telomerase reverse transcriptase (hTERT). As hTERT elongates telomeres and protects mitochondrial DNA (mtDNA) from oxidative damage, its inhibition could lead to shorter telomeres and/or increased mitochondrial dysfunction. Leukocyte telomere length (LTL) and mtDNA alterations are biomarkers of cellular aging, and have been implicated in aging and age-related diseases.

The objective of my research was to compare HEU and HIV-unexposed uninfected (HUU) children at birth and in early life, with respect to their LTL and blood mtDNA content, and investigate relationships with *in utero* cART exposure.

I measured LTL and blood mtDNA content in 324 HEU and 306 HUU children between 0-3y of age. I found that exposure to maternal cART did not affect LTL at birth, as it was similar in both groups. However, mtDNA content was higher among HEU children, particularly those exposed to boosted-protease inhibitor (PI/r) cART. This increase in mtDNA persisted at least up to age three. Additionally, maternal smoking during pregnancy affected both LTL and mtDNA content at birth.

Given these effects of cART on children's mtDNA, I aimed to further characterize how various cART regimens affect mitochondrial health using an in vitro human cell culture model. I also investigated the potential mitochondrial protection conferred by hTERT. I found that dolutegravir (DTG)-containing regimens negatively affected mitochondria, decreased cell proliferation and increased apoptosis. PI/r-containing regimens also affected certain mitochondrial parameters, but this effect was mitigated by mitochondrial hTERT while that of DTG was not.

DTG is increasingly used worldwide, including in pregnancy. These novel findings merit further investigations to evaluate the long-term safety of newer ARV exposure, and the predictive value of these biomarkers on HEU health outcomes. Together, this knowledge could inform treatment guidelines.

Lay Summary

Treatment of women living with HIV during pregnancy has dramatically reduced the risk of mother-to-child transmission. However, many HIV drugs can cross the placenta and may have unwanted effects in the unborn child. In some instances, HIV drugs can affect the DNA of cells, something that could accelerate aging, and lead to disease. Among uninfected children born to mothers living with HIV who, as recommended, took HIV drugs during their pregnancy, I found no evidence that treatment damaged the cell's DNA. However, certain drugs, including a newer drug, increased the amount of a specific type of cellular DNA. This effect in newborns persisted at least until age three. My research strongly suggests that all HIV drugs do not have the same effects and that more research is needed to ensure that HIV drugs taken during pregnancy are safe in the long term, for both mother and child.

Preface

The contents of this dissertation are my original work. All experiments were designed and conducted by me in conjunction with my supervisor, Dr. Hélène Côté, and the guidance of my supervisory committee. All conducted research was previously approved by the University of British Columbia Research Ethics Board (H03-70356, H04-70540, H07-03136, H08-02018, and H18-03076). Written informed consent was obtained from the parents/guardians of all study participants, except for anonymous control children, for whom this was not required.

Chapter one is an introduction to my thesis, beginning with a review of the relevant literature and ending with the main objectives and overarching hypothesis of my research. Considering that a major portion of my research focuses on biomarkers of cellular aging among human immunodeficiency virus (HIV)-exposed uninfected (HEU) children born to women living with HIV (LWH), the introduction has been tailored to focus specifically on HIV and combination antiretroviral therapy (cART) in the context of pregnancy. To provide an overview of the evolution of treatment guidelines over the years for pregnant women LWH, I created Table 1.2 using data obtained from the World Health Organization' recommendations. Likewise, to provide an overview of HIV and pregnancy in Canada, I used data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP) to make figures 1.3, 1.4, and 1.5, showing the number of pregnancies per year, the timing of therapy initiation during pregnancy, and the type of cART regimens received during pregnancy, respectively, between 1990 and 2016. Permission to use CPHSP data for these figures was granted by Drs. Ari Bitnun and Laura Sauve via email.

A version of **chapter two has been published** as Ajaykumar A, Soudeyns H, Kakkar F, Brophy J, Bitnun A, Alimenti A, Albert AYK, Money DM, Côté HCF, and the CIHR Team in Cellular Aging and HIV Comorbidities in Women and Children (CARMA) (2018), "**Leukocyte Telomere Length at Birth and During the Early Life of Children Exposed to but Uninfected With HIV After In Utero Exposure to Antiretrovirals"**, *The Journal of Infectious Diseases*, 217 (5):710-720. I am the first author on this manuscript. For the purposes of this thesis chapter, the only change made from that of the published article is to the manuscript's introduction, which has been modified to avoid repetition. Supplementary material (tables and figures) from this publication have been incorporated into the main text, as appropriate. As part of this study, I extracted all relevant demographic and clinical information of study participants from the cohort databases, and conducted all telomere assays using a monochrome multiplex qPCR (MMqPCR) assay recently optimized by our laboratory. I also conducted all cross-sectional univariate and multivariable statistical analyses, and was responsible for interpreting the longitudinal data analysis performed by our biostatistician, Dr. Albert. I prepared all figures but one (Figure 2.7), and wrote the manuscript. Drs. Soudeyns, Kakkar, Brophy, Bitnun, Alimenti and Money contributed to the design of the study, to clinical data collection, and they edited the manuscript. Dr. Côté designed the study, oversaw the laboratory data collection, and edited the manuscript.

Details regarding the MMqPCR assay used in this study have been published as Hsieh AYY[*], Saberi S[*], Ajaykumar A, Hukezalie K, Gadawski I, Sattha B, and Côté HCF (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. I am the second author on this manuscript. I conducted

some of the MMqPCR assays to measure TL across different tissue specimen and validated the assay against the previously used monoplex qPCR method. I also critically reviewed and edited the final version of the manuscript. Mr. Hsieh and Dr. Saberi conducted 70% of the assays and wrote the manuscript. Mr. Hukezalie contributed to data collection and validation. Ms. Gadawski and Ms. Sattha processed all biological specimens and contributed to assay validation. Dr. Côté designed the study, oversaw the laboratory data collection, and edited the manuscript. This robust and high-throughput method is now used extensively in our laboratory to measure TL for many studies, including the study in chapter three of this thesis.

Chapter three: "The effects of *in utero* exposure to maternal smoking on infant telomere length" is a manuscript ready to be submitted to a peer-reviewed journal. I am the first author on this manuscript, co-authored by Saberi S, Alimenti A, Sauve L, Forbes JC, Van Schalkwyk J, Money DM, Côté HCF, and the CIHR Team in Cellular Aging and HIV Comorbidities in Women and Children (CARMA). This study was conceptualized following the results described in chapter two, whereby HIV-unexposed uninfected (HUU) infants born to mothers who smoked during pregnancy had longer leukocyte telomere length (LTL) at birth. Given this counter-intuitive finding, I decided to investigate the effect of smoking on TL in several other infant tissues, including umbilical cord blood and cord tissue, placenta, and mouth epithelial cells. I conducted all assays, analyzed the data, prepared figures and wrote the manuscript. Drs. Saberi, Alimenti, Sauve, Forbes, Van Schalkwyk and Money contributed to the design of the study, to clinical data collection, and they edited the manuscript. Dr. Côté designed the study, oversaw the laboratory data collection, and edited the manuscript.

Chapter four: "Blood mtDNA levels are persistently elevated from birth into early life in cART-exposed HIV-exposed uninfected (HEU) children" is a **manuscript that has been submitted** for publication and is currently under review. I am the first author on this manuscript, co-authored by Zhu M, Soudeyns H, Kakkar F, Brophy J, Bitnun A, Alimenti A, Albert AYK, Money DM, Côté HCF, and the CIHR Team in Cellular Aging and HIV Comorbidities in Women and Children (CARMA). Once again, the manuscript's introduction has been modified to avoid repetition in this chapter. This study included the same cohort of HEU and HUU children described in chapter two. I conducted all mtDNA assays, analyzed the data, prepared figures and wrote the manuscript. Ms. Zhu contributed to MMqPCR data collection, and edited the manuscript. Dr. Albert conducted the longitudinal data analyses, prepared corresponding figures, and edited the manuscript. Drs. Soudeyns, Kakkar, Brophy, Bitnun, Alimenti and Money contributed to the design of the study, to clinical data collection, and they edited the manuscript. Dr. Côté designed the study, oversaw the laboratory data collection, and edited the manuscript.

Chapter five: "Pharmacological concentrations of cART affect mitochondrial and cellular health in a transformed cell culture model: protective role of human telomerase reverse transcriptase" is a manuscript in preparation, the title of which is currently tentative. I am the first author on this manuscript, co-authored by Hsieh A, Caloren L, Uday P, Pattanshetti R, Thompson C, Wong JMY, and Côté HCF. I helped design the study along with Côté, HCF and Wong, JMY, carried out all cell culture experiments, collected cells for flow cytometry and DNA extraction, and conducted mtDNA content assays. Mr. Hsieh made significant contributions to the optimization of the flow cytometry panel, and conducted some of the flow

cytometry experiments. Mr. Caloren assisted with the flow cytometry experiments. Ms. Uday and Ms. Pattanshetti assisted with the cell culture experiments, DNA extractions and mtDNA content assays. Finally, I conducted all data analyses, prepared the figures and wrote the manuscript.

Chapter six is a summary of the overall conclusions of my thesis, including contributions and significance to the field, and future directions.

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List of Abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immune deficiency syndrome
ALT	Alternate lengthening of telomeres
APV	Amprenavir
ART	Antiretroviral therapy
ARV	Antiretrovirals
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATZ	Atazanavir
AZT	Zidovudine
CARMA	Children and Women: Antiretrovirals and Markers of Aging
cART	Combination antiretroviral therapy
cDNA	Complementary DNA
Cmax	Maximum plasma concentration
CMV	Cytomegalovirus
CPHSP	Canadian Perinatal HIV Surveillance Program
CTN	Canadian HIV Trials Network
d4T	Stavudine
DAPI	4′,6-diamidino-2-phenylindole
ddC	Zalcitabine
ddI	Didanosine
DLV	Delavirdine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOR	Doravirine
DRV	Darunavir
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
ER	Endoplasmic reticulum
ETC	Electron transport chain
ETR	Etravirine
EVG	Elvitegravir

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPV	Fosamprenavir
FTC	Emtricitabine
GA	Gestational age
HCV	Hepatitis C Virus
HEU	HIV-exposed uninfected
HIV	Human immunodeficiency virus
hTERT	Human telomerase reverse transcriptase
hTR/TERC	Human telomerase RNA component
HUU	HIV-unexposed uninfected
IDV	Indinavir
InSTIs	Integrase nuclear strand transfer inhibitors
IQR	Interquartile range
LPV/r	Ritonavir-boosted Lopinavir
LTL	Leukocyte telomere length
LWH	Living with HIV
MFI	Median fluorescence intensity
MMP	Mitochondrial intermembrane potential
MMqPCR	Monochrome multiplex quantitative polymerase chain reaction
MTCT	Mother-to-child transmission
mtDNA	Mitochondrial DNA
MTS	Mitochondrial-targeting signal
MVC	Maraviroc
NFV	Nelfinavir
NIH	National Institutes of Health
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs/NtRTIs	Nucleoside/nucleotide analog reverse transcriptase inhibitors
NS	Non-smoker
NVP	Nevirapine
OXPHOS	Oxidative phosphorylation
PACTG	Pediatric AIDS Clinical Trials Group
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PHAC	Public Health Agency of Canada
PI	Protease inhibitor
PI/r	Ritonavir-boosted protease inhibitor
PLWH	People living with HIV
POLγ	Mitochondrial polymerase gamma

pVL	Plasma viral load
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RAL	Raltegravir
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPV	Rilpivirine
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RTV	Ritonavir
S	Smoker
SGA	Small for gestational age
SQV	Saquinavir
T-20	Enfuvirtide
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TL	Telomere length
TPV	Tipranavir
tRNA	Transfer RNA
WB	Whole blood
WHO	World Health Organization
WT	Wildtype

Acknowledgements

I would first like to express my sincere gratitude to my supervisor, Dr. Hélène Côté, for the continuous support of my PhD study and related research, and for her patience, motivation, and immense knowledge. Her mentorship has been instrumental in my all-rounded development as a student and scientist. I could not have imagined having a better advisor for my PhD study. I would also like to thank my supervisory committee: Drs. Mark Scott, Judy Wong, Soren Gantt and Mel Krajden, for their insightful comments and encouragement, their time and commitment to my projects, and for their hard question which incented me to widen my research from various perspectives. Special thanks to Dr. Haydn Pritchard for his support and advice throughout my graduate degree.

I would also like to thank all members of the Côté lab, past and present, for their friendship, support, and aid during my time here. To my peers: Anthony, Sara, Matt, Marta, Adam, Micah, Marie-Soleil, and Rachel: thank you for your constant support and for all the fun we have had in the last five years. I wish you the very best in your future endeavours. It is only befitting that I extend a special thank you to Anthony: your assistance and input throughout my studies, together with your unwavering friendship and moral support were invaluable, and in you I have gained a lifelong friend. To Izabelle and Beheroze: I thank you from the bottom of my heart for the countless hours you have spent training, teaching and troubleshooting with me. Izabelle, the love and care you have showered on each one of us in unparalleled, and I thank you for being the wonderful person that you are. To all the undergraduate students who have been a part of our lab: Mayanne, Omair, Elana, Aïda, Colin, Brian, Parmeet, Aya, Prakruti, Rutuja,

Loïc, Nancy, and Mahtab: I have enjoyed working with all of you and will cherish the friendships I have made. Thank you for being the life of the "Côté lab socials" and I sincerely hope the experiences you have gained will serve you well going forward.

Finally, I would like to thank my friends and family for their continuous support, love and guidance. To my partner and best friend, Katerina: thank you for being my better half. Your love, care and affection is unmatched. I cannot express how grateful I am for your patience, understanding and kindness throughout these years. I hope I have been able to reciprocate the affection you have shown me. To my dear friend, Aarya: thank you for 10+ years of wonderful friendship, and for caring for me like your own. You have become an invaluable part of my life and I wish you the best. Words cannot express my gratitude to my family. To my parents, Indu and Ajaykumar: this would not have been possible without you. Your unconditional love and support have shaped me into the strong and confident man I am today. I am forever indebted to you and can only hope that I make you proud every day. To my siblings, Aniketh and Ananyaa: thank you for being the best brother and sister a person could ask for. The affection, love and constant encouragement you have shown towards your big brother has always been appreciated, and I sincerely hope I have set a good example for you. The love, generosity and sacrifices you have all shown towards my success have been nothing short of inspirational!

To my parents, Indu and Ajaykumar

Chapter 1: INTRODUCTION

1.1 Epidemiology of HIV/AIDS

As of 2017, an estimated 37 million people were living with human immunodeficiency virus (HIV) globally, of which 17.4 million (47%) were women of childbearing age and 1.8 million (5%) were children below the age of fifteen [1]. Approximately 90% of all HIV-infected children acquired the virus perinatally from their mothers. Management of HIV with antiretroviral therapy (ART) has prevented the transition to acquired immune deficiency syndrome (AIDS), reduced HIV-related morbidity and mortality, and increased the lifespan of people living with HIV (PLWH). The widespread use of ART has also drastically reduced the rates of HIV transmission. In 2017, 21.7 million (59%) PLWH accessed ART, reducing the number of new HIV infections to 1.8 million, compared to 3.4 million during the peak of the epidemic in 1996. At the same time, the number of deaths associated to AIDS-related illnesses has reached an all-time low of 940,000, compared to 1.9 million in 2004 [1].

In Canada, an estimated 72,200 people were living with HIV (LWH) by the end of 2016, including 9,090 who were unaware that they had HIV [2]. Of the 63,110 confirmed cases, approximately 14,520 (23%) were women [3]. While the number of new HIV cases reported per year has remained relatively stable since 2002, there were an estimated 2,344 new HIV infections in 2016, representing an 11.6% increase from the 2,100 cases reported in 2015 [3,4]. This increase may be partially attributed to advancements in HIV surveillance and/or HIV testing across the country. The number of new HIV infections among children (<15y) has also remained stable over the years, with only 14 documented cases of vertical HIV transmission reported since 2011 [5]. Currently, there are about 250 children receiving care for HIV in Canada [4–6].

1.2 Combination Antiretroviral Therapy (cART)

The first antiretroviral (ARV) drug to treat HIV-infection, zidovudine (AZT), was approved in 1987. Since then, several classes of ARVs have evolved, each targeting a different stage of the HIV replication cycle. The strategy of combining two or more drug classes to block multiple stages of the HIV life cycle became known as combination antiretroviral therapy (cART) and has considerably changed the landscape of HIV treatment. Since 1996, cART has become the gold standard to treat HIV infection. The development of new ARVS over time has extensively reduced viremia, while improving survival, tolerability, and safety compared to early therapies [7,8].

1.2.1 Classes of cART, mechanisms of action and toxicities

Based on their mechanism of action, six major classes of ARV drugs have been approved by the United States Food and Drug Administration (FDA) for the treatment of HIV. Nucleoside/nucleotide analog reverse transcriptase inhibitors (NRTIs/NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) remain the more established and widely used drug classes. Newer classes such as integrase nuclear strand transfer inhibitors (InSTIs) are steadily making inroads into current first-line regimen, due to their enhanced and rapid antiviral potency [9,10], while others such as fusion inhibitors and entry inhibitors are used sparingly. Currently, cART is typically administered as a combination of two NRTIs (“backbone”) and one drug from a PI, NNRTI or InSTI (“base”) [11].

1.2.1.1 Nucleoside/nucleotide analog Reverse Transcriptase Inhibitors (NRTIs/NtRTIs)

NRTIs are structural analogs of the four naturally occurring nucleosides: adenosine, thymidine, cytidine and guanosine. However, they lack a 3`-hydroxyl group on the deoxyribose moiety that is required for incorporation of nucleosides into the DNA chain through phosphodiester bond formation. Hence, by inducing chain termination, they act as competitive inhibitors of HIV reverse transcriptase (RT), preventing the reverse transcription of viral RNA to DNA required for integration into the host genome [12]. Although highly important in HIV therapy, NRTIs can affect the human telomerase and mitochondrial polymerase gamma (POLγ) enzymes *in vitro*, as will be discussed in subsequent sections. This can result in cellular and mitochondrial toxicities that may have long-term clinical manifestations. Indeed, several NRTIs have been linked with adverse clinical events, with symptoms ranging from milder conditions such as nausea, headaches, insomnia, dizziness and fatigue, to more serious, even life-threatening toxicities such as neuropathy, lactic acidosis, hepatotoxicity, and renal failure [13].

1.2.1.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs on the other hand are non-competitive inhibitors of HIV RT. They affect the RT's ability to handle nucleosides by binding to an allosteric site of the enzyme and inducing conformational changes [14]. NNRTIs bind to the NNRTI-binding pocket on the HIV RT and by doing so, lock the adjacent DNA polymerase active site in an open conformation, thereby preventing subsequent nucleoside polymerization. NNRTI adverse effects include skin and neuropsychiatric effects, for example rashes, insomnia and abnormal dreams [13,15–17]. In addition to these adverse effects, in the context of pregnancy, NVP has been associated with serious hepatotoxicity [15,18,19], while possible teratogenic effects were reported for EFV

[20,21]. For these reasons, the drugs were avoided during pregnancy in Canada but were still widely used in many countries.

1.2.1.3 Protease Inhibitors (PIs)

PIs target the viral protease enzyme that is necessary for the proteolytic cleavage of HIV polypeptide precursors required for the production of mature virions upon budding from the host cell membrane [22]. Most PIs are rapidly metabolized by the liver, via the cytochrome P450 enzymes. Ritonavir (RTV) however is a strong inhibitor of liver cytochrome p450 enzymes. It thereby slows the metabolism of other PIs, hence "boosting" their pharmacological availability [23], and is commonly used as a potentiating agent in PI-based regimens. Although PIs are generally well tolerated, adverse reactions include diarrhea, nausea, headache and more severe clinical toxicities such as hepatotoxicity, nephrotoxicity, lipodystrophy, pancreatitis, insulin resistance, and other metabolic complications [13,24]. The mechanisms of PI-mediated toxicities will be discussed in subsequent sections.

1.2.1.4 Integrase nuclear Strand Transfer Inhibitors (InSTIs)

InSTIs are the newest classes of ARVs. They inhibit HIV integrase which catalyzes the insertion of viral cDNA into host nuclear DNA, a process known as strand transfer [25]. InSTIs bind to Mg^{2+} metal ions at the active site of integrase, thereby acting as competitive inhibitors. Raltegravir (RAL), dolutegravir (DTG) and elvitegravir (EVG) are three currently FDA-approved InSTIs. Although InSTIs were initially used to treat patients with HIV drug resistance, they are now increasingly used in first-line cART regimens due to their tolerability and ability to rapidly suppress viremia [26–29]. The latter has made InSTIs an attractive option to treat

pregnant women presenting with HIV late in pregnancy [30,31]. However, data on the long-term safety of InSTIs, especially in the context of pregnancy, are limited and warrant further investigations. Adverse neuropsychiatric effects have been reported among individuals treated with InSTIs [32–34]. Notably, a recent study from Botswana reported an early signal for increased prevalence of neural-tube defects among children exposed to maternal DTG-based cART since conception [35].

1.2.1.5 Fusion and Entry Inhibitors

These classes of drugs block receptors at the cell surface and thereby inhibit the fusion and entry of HIV into the host cell [36]. Enfuvirtide (T-20), a fusion inhibitor, and maraviroc (MVC), an entry inhibitor, are the only two FDA approved drugs in these classes. However, they are used selectively, on account of issues associated with HIV tropicity, cost, inconvenience of administration, allergies, and liver toxicities. Due to insufficient data regarding the safety and toxicity of these drugs during pregnancy, they are rarely prescribed to pregnant women.

The list of all ARVs approved by the FDA for the treatment of HIV, along with their ability for trans-placental transfer, is described below in Table 1.1. All FDA-approved ARVs were also approved by the Public Health Agency of Canada for the treatment of PLWH in Canada.

Table 1.1. List of all ARVs approved by the FDA for HIV treatment.

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)			
Generic Name	**Abbreviation**	**Approval date**	**Placental transfer[a]**
Zidovudine	AZT, ZDV	Mar-87	High
Didanosine	ddI	Oct-91	Moderate
Zalcitabine[b]	ddC	Jun-92	Not used
Stavudine	d4T	Jun-94	High
Lamivudine	3TC	Nov-95	High
Abacavir	ABC	Dec-98	High
Tenofovir disoproxil fumarate	TDF	Oct-01	High
Emtricitabine	FTC	Jul-03	High
Tenofovir alafenamide	TAF	Nov-15	High
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
Generic Name	**Abbreviation**	**Approval date**	**Placental transfer**
Nevirapine	NVP	Jun-96	Moderate
Delavirdine	DLV	Apr-97	No data
Efavirenz	EFV	Sep-98	Moderate
Etravirine	ETR	Jan-08	Moderate
Rilpivirine	RPV	May-11	Insufficient data
Doravirine	DOR	Aug-18	No data
Protease Inhibitors (PIs)			
Generic Name	**Abbreviation**	**Approval date**	**Placental transfer**
Saquinavir	SQV	Dec-95	Undetectable/None
Ritonavir	RTV	Mar-96	Low
Indinavir	IDV	Mar-96	Undetectable/None
Nelfinavir	NFV	Mar-97	Undetectable/Low
Amprenavir	APV	Apr-99	No data
Ritonavir-boosted Lopinavir	LPV/r	Sep-00	Low
Atazanavir	ATZ	Jun-03	Low
Fosamprenavir	FPV	Oct-03	Low/Moderate
Tipranavir	TPV	Jun-05	Insufficient data
Darunavir	DRV	Jun-06	Low

Integrase Strand Transfer Inhibitors (InSTIs)			
Generic Name	**Abbreviation**	**Approval date**	**Placental transfer**
Raltegravir	RAL	Oct-07	Very High
Dolutegravir	DTG	Aug-13	No data
Elvitegravir	EVG	Sep-14	No data
Fusion Inhibitors			
Generic Name	**Abbreviation**	**Approval date**	**Placental transfer**
Enfuvirtide	T-20	Mar-03	Undetectable/None
Entry Inhibitors			
Generic Name	**Abbreviation**	**Approval date**	**Placental transfer**
Maraviroc	MVC	Aug-07	Undetectable/None

[a]Based on cord-to-maternal plasma (C:M) accumulation ratios obtained from McCormack and Best [37] and Else LJ *et al.* [38]

[b]Zalcitabine was removed from the market in 2006 due to associated toxicities.

1.3 Aging with HIV/cART

Despite the success of cART, there is a growing body of evidence suggesting that PLWH develop age-related diseases such as cardiovascular disease, Alzheimer's disease, osteoporosis, diabetes and cancer earlier in life [39,40]. In addition to this, numerous pathologies consistent with mitochondrial dysfunction including, hyperlactatemia and lactic acidosis, lipodystrophy, myopathy and nephropathy have been reported among ART-treated PLWH [13,41,42]. It is now widely hypothesized that PLWH, even when successfully treated, experience premature or accelerated aging.

Two key theories have evolved over the years to explain the aging process: the telomere shortening/telomerase inhibition cellular replicative senescence theory of aging [43–45], and the mitochondrial oxidative stress theory of aging [46,47]. The following sections will elaborate on these theories, and the role of HIV and cART in the aging process.

1.3.1 Telomere shortening, telomerase inhibition, HIV and cART

Telomeres consist of repetitive TTAGGG nucleotide sequences and protein binding complexes that together protect the ends of eukaryotic chromosomes from damage and/or fusion [48,49]. Telomere length (TL) in humans varies from 5-15 kilobases, and is maintained by the enzyme telomerase, a ribonucleoprotein complex consisting of two core subunits: the human telomerase RNA template (hTR or TERC) and the catalytic subunit human telomerase reverse transcriptase (hTERT) [50]. Activated immune cells [51,52], germ cells, embryonic stem cells, placental cells [53] and most human cancers [54] express telomerase, resulting in telomere length maintenance and/or slower TL attrition in these cells. However, most somatic cells do not express telomerase and consequently, TL shortens with each cellular division until it reaches a critical point called the Hayflick limit, beyond which cell division is inhibited and cells enter a stage of replicative senescence [54]. TL thus provides an account of a cell's replication history and/or its remaining replicative potential, and is a biomarker of aging. Shorter leukocyte TL (LTL) is correlated with mortality [55], and has been linked to several of the aforementioned age-related diseases[56–58].

In the context of HIV, several cohort studies have reported shorter LTL in PLWH compared to HIV-uninfected controls [59–62]. Potential mechanisms include HIV-mediated chronic immune activation, oxidative stress and inflammation [40,63,64]. Indeed, oxidative stress can induce telomere shortening in human fibroblast cells through single-strand breaks in telomeric DNA [65]. Additionally, HIV may also cause telomere shortening by decreasing telomerase activity in cells. To this extent, a few studies reported undetectable and/or significantly reduced telomerase activity in peripheral blood lymphocytes [66] and uninfected

hematopoietic progenitor cells isolated from PLWH [67], as well as in activated CD4+ T cells infected with HIV *in vitro* [68]. Some ARVs could also modulate telomere attrition and contribute to the accelerated aging phenotype reported in PLWH. Given its high structural and functional homology with HIV RT [69], telomerase could be affected by NRTIs. In this regard, NRTIs have been shown to inhibit telomerase activity *in vitro* [70–72] and shorten telomeres in immortalized telomerase-dependent cell lines [73,74]. PIs and some older NRTIs such as AZT and d4T are known to induce oxidative stress *in vitro*, through the production of reactive oxygen species (ROS) [75–77], which could also damage and shorten telomeres. Although the association between shorter LTL and HIV infection is well established, the relationship with cART exposure *in vivo* has not been clearly demonstrated. In fact, some studies have shown that cART-treated individuals have longer LTL or slower LTL attrition compared to treatment-naïve PLWH [78,79], suggesting that cART may exert an overall beneficial effect on telomeres, likely through reduced chronic immune activation.

Despite this, it is imperative to investigate the long-term effects of ARV exposure in the HIV-infected population, especially early on in life, including in young HIV-infected children as well as HIV-exposed uninfected (HEU) children exposed to cART *in utero.*

1.3.2 Mitochondrial oxidative stress theory of aging

Mitochondria are double-membraned cellular organelles that play a central role in eukaryotic cellular metabolism [80]. The inner membrane of mitochondria contains the enzyme complexes of the mitochondrial respiratory chain (also known as the electron transport chain, ETC), and serves as the primary site for adenosine triphosphate (ATP) production via oxidative

phosphorylation (OXPHOS). Mitochondria are also unique in that they contain their own DNA, the mitochondrial DNA (mtDNA). MtDNA is a 16.5 kb circular, double-stranded DNA molecule. It is replicated by mitochondrial polymerase gamma (POLγ), and such replication can occur independent of both cellular division and mitochondrial proliferation. MtDNA encodes 13 proteins that are subunits of the ETC complexes, as well as two ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) that are essential for translation of mitochondrial proteins [81,82]. MtDNA is maternally inherited (although a recent publication suggests the possibility of paternal inheritance [83]), and in most individuals the majority of molecules contain the same conserved sequences (homoplasmy). The number of mitochondria per cell, as well the cell's mtDNA content, varies greatly depending on the cell type and the stage of development. Highly metabolically active tissues such as heart, muscle and brain have a high mtDNA content [84,85]. In the general population, both elevated and reduced mtDNA levels have been implicated in disease and aging [85].

The production of excess endogenous ROS forms the backbone of the original oxidative stress theory of aging [86–88]. Mitochondria play a central role in this process, since they are both a major producer and target of ROS. The close proximity of mtDNA to the ROS-generating ETC, together with its lack of a protective histone core, the mitochondria's simple DNA repair machinery, and the low fidelity of POLγ, makes mtDNA extremely susceptible to oxidative damage and mutations [86–88]. This in turn could impair mitochondrial protein assembly, alter mitochondrial function, and ultimately lead to cell death [89]. It was originally assumed that mitochondrial ROS would increase the acquisition of new (somatic) mtDNA mutations. However, more recent studies suggest that somatic mutations may be predominantly introduced

as a result of POLγ errors during mtDNA replication [90,91], which would then be expanded with subsequent mtDNA replications. The accumulation and/or clonal expansion of such somatic mtDNA mutations over time would ultimately result in increased mtDNA heteroplasmy, and subsequent mitochondrial dysfunction and disease. This is now the more widely accepted theory of mitochondrial aging [87,92].

1.3.2.1 Mitochondrial aging with HIV and cART

As mentioned previously, several pathologies related to mitochondrial toxicity and dysfunction have been reported among ARV-treated PLWH [13,41,42], particularly with early generation NRTIs. NRTIs can inhibit the mitochondrial POLγ that replicates mtDNA, thereby depleting mtDNA and altering mitochondrial function [93]. In addition, NRTI pressure may affect the fidelity of POLγ, thereby increasing mtDNA mutations/deletions. The latter has been reported in HIV-infected individuals treated with NRTI-containing regimens [94–98]. NRTIs have also been shown to increase oxidative stress *in vitro* [99–102], reduce mtDNA content in cultured cells [103–106], and cause oxidative DNA damage in animal and human studies [107–110]. Several *in vivo* studies have reported decreased mtDNA content and mitochondrial enzyme activities among treatment-naïve as well as NRTI-treated PLWH, compared to uninfected peers [111–127]. Although ARV treatment generally improved mtDNA levels in these individuals, the use of older NRTIs such as d4T, ddI and AZT was associated with mtDNA depletion. This effect was at least partially reversible, as mtDNA levels rebounded following discontinuation/switch of therapy [111,128–132]. Newer NRTIs such as TDF, ABC, and FTC have been shown to exert lesser mitochondrial toxicity, and are relatively safe [133–136].

PIs, like some NRTIs, are known to increase ROS in cultured cells [137–142], which could in turn affect mtDNA and mitochondrial function as described previously. Likewise, HIV proteins have also been shown to increase ROS, induce apoptosis, deplete mtDNA content, and alter mitochondrial function *in vitro* [143–152]. Taken together, exposure to NRTIs, PIs and/or HIV proteins could result in the accumulation of damaged and dysfunctional mitochondria within cells. Mitochondria are highly dynamic organelles that are capable of undergoing fusion, fission and autophagy for their maintenance, as well as for protection against persistent mtDNA damage [153,154]. Alterations in mitochondrial morphology and/or mass could be indicative of mitochondrial health and may reflect a compensatory mechanism to preserve mitochondrial function and ensure cell survival. For instance, mitochondrial autophagy (mitophagy) can eliminate damaged mitochondria and prevent cellular apoptosis, but this could further decrease overall cellular mtDNA levels. In line with this, one study reported abnormal hepatic cell mitochondrial morphology, increased mitochondrial mass, and upregulated mitophagy as a protective mechanism against mitochondrial toxicity induced by treatment with EFV [155]. On the other hand, damaged/dysfunctional mitochondria could elicit compensatory biogenesis and mtDNA replication, which could in turn increase overall mtDNA content [156]. However, this may promote the clonal expansion of pre-existing mutations, thereby increasing mitochondrial heteroplasmy and further exacerbating mitochondrial health. Consequently, both mtDNA quantity and quality could contribute to overall mitochondrial function, and may act as biomarkers of mitochondrial health.

Figure 1.1 describes the inter-relationship(s) between HIV, ARVs, TL, telomerase and mtDNA that may contribute towards accelerated cellular aging.

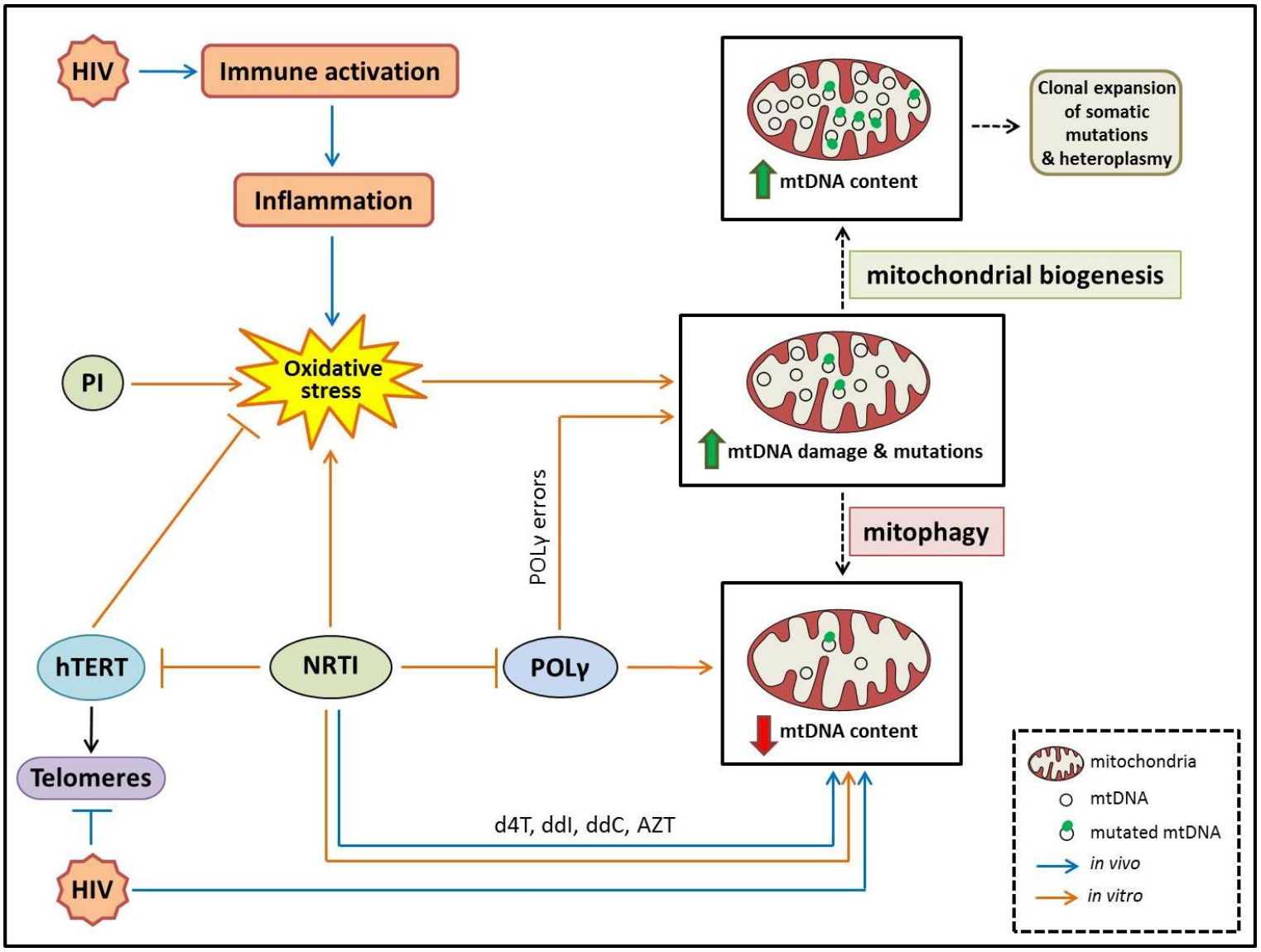

Figure 1.1. Relationship(s) between HIV, ARVs, Telomeres, hTERT and mtDNA

1.3.2.2 Telomerase and the mitochondrion

Aside from its canonical function in telomere maintenance, hTERT has extra-telomeric roles related to protection of mitochondria and mtDNA from oxidative stress-induced damage [157–161]. hTERT contains an N-terminal mitochondrial-targeting signal (MTS) and translocates from the nucleus to the mitochondria in response to increased oxidative stress [161,162]. Although the exact mechanism of hTERT protection is not fully elucidated, mitochondrial translocation of hTERT has been shown to reduced ROS generation, improve respiratory chain function, increase mitochondrial intermembrane potential (MMP), reduce mtDNA damage, and improve overall mitochondrial function *in vitro* [159–161,163].

As mentioned previously, NRTIs can inhibit hTERT activity *in vitro*[70–72]. Given this, similar effects could be expected *in vivo*. In this regard, a recent study reported a compensatory increase in hTERT mRNA expression in relation to NRTI-based ARV-induced mitochondrial toxicity [164]. As hTERT is expressed in stem cells, germ cells and placenta, cART-associated mitochondrial toxicity and telomerase (i.e. hTERT) inhibition in the context of pregnancy could potentially result in irreversible changes during early fetal development.

The following sections will discuss the impact of HIV and cART in pregnancy, highlighting the evolution of treatment guidelines for pregnant women LWH, adverse outcomes in pregnancy and consequences of *in utero* HIV/cART exposure on the health and development of HEU children.

1.4 HIV and cART in pregnancy

As mentioned previously, 90% of children LWH acquire the virus from their mothers. Vertical HIV transmission mainly occurs when the newborn is exposed to maternal body fluids during childbirth [165], but can also occur prenatally via placental transmission [165,166], as well as postnatally during breastfeeding [167]. Vertical HIV transmission has been significantly curtailed, with rates of mother-to-child-transmission (MTCT) falling from as high as 45% during the pre-ART era (15-30% in non-breastfeeding and 20-45% in breastfeeding populations) [167], to ~8% with AZT monotherapy in pregnancy [168], and below 5% worldwide with the use of current cART regimens in pregnancy [169].

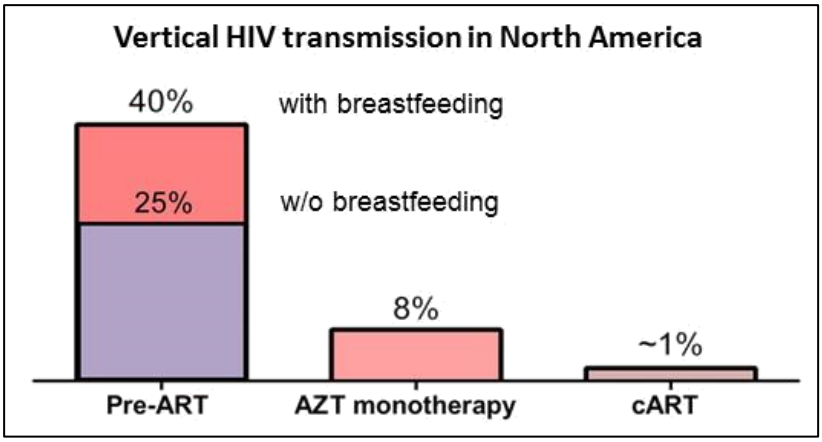

Figure 1.2. Vertical HIV transmission rates in North America [170]

Over 80% of pregnant women LWH worldwide now have access to cART and consequently, the number of new HIV infections among children has declined by 35%, from 270,000 in 2010 to 180,000 in 2017 [1]. Women LWH give birth to ~1.8-2.0 million HEU children every year, most of them exposed to maternal cART, and this number continues to rise. In some parts of southern Africa, HEU infants represent 30% of all children born [171]. As of 2017, there were an estimated 14.8 million HEU children worldwide. In Canada, since 2009, the

number of reported HIV-exposed births has ranged from 200-250 per year, with a high of 263 in 2016 [4]. Of these 263 perinatally HIV-exposed infants, only a single case of HIV transmission was confirmed, in a mother not receiving cART [4]. Since 2011, >94% of all pregnant women LWH in Canada received pre- and post-natal cART and as a result, there have only been 14 documented cases of vertical HIV transmissions [5]. It is noteworthy that none of the 14 reported cases in this period were from British Columbia, one of only two provinces (the other being Yukon Territory) where 100% of pregnant women received antenatal cART, ~97% of them for at least 4 weeks before delivery [5].

1.4.1 Evolution of treatment guidelines in pregnancy

The very first report of HIV treatment during pregnancy came from the Pediatric AIDS Clinical Trials Group protocol 076 (PACTG 076), demonstrating that AZT monotherapy initiated between 14-34 weeks of pregnancy reduced the risk of MTCT by 67.5% (from 25.5% to 8.3%) [168]. This gave rise to the first universal guideline for the treatment of HIV-infected pregnant women with oral AZT monotherapy starting after the first trimester of pregnancy (>13w), along with an intravenous dose during labour. Newborns were also given oral AZT prophylaxis for the first six weeks of life, and this combined maternal-infant treatment strategy was adopted as the standard of care for pregnant women LWH until the late 1990s. The first WHO guidelines published in 2001 modelled this strategy, but recommended initiation of ART only if CD4 cell counts fell below 200 cells/μL [172]. Additionally, treatment options were expanded to include a second NRTI 3TC, in combination with AZT. Table 1.2 describes the evolution of WHO guidelines for treating pregnant women LWH and preventing HIV infection in infants.

Table 1.2. WHO treatment guidelines for pregnant women LWH and their infants (prophylaxis).

Year	Guidelines for initiation	Mother			Infant
		When to initiate	Recommended First-line regimen(s)	Recommended Second-line regimen(s)	Recommended prophylaxis started at birth
2001	CD4 cell count **<200 cells/µL**	After 1st trimester (>13w)	**AZT**, or **AZT+3TC**	NVP	**AZT for 6w**, or **AZT+3TC for 1w**
2004	CD4 cell count **<200 cells/µL**	As soon as possible As soon as possible	**AZT+3TC+NVP** **or** **d4T+3TC+NVP**	AZT+3TC+EFV or d4T+3TC+EFV (after 1st trimester) Alternate second-line: SQV/r or NFV with AZT+3TC or d4T+3TC	**Twice-daily AZT for 1w**, or **Once-daily NVP** or **once-daily NVP+AZT for 1w**
2006	CD4 cell count **<200 cells/µL**	As soon as possible after 1st trimester (>13w)	**AZT+3TC+NVP** **or** **AZT+3TC+EFV**	AZT+3TC+SQV/r or AZT+3TC+LPV/r	**Twice-daily AZT for 1w, or for 4w** (if maternal cART started <4w before delivery), or **Single-dose NVP+AZT for 1w**
2010	CD4 cell count **<350 cells/µL**	As soon as possible after 1st trimester (>13w)	**AZT+ 3TC+NVP or TDF+3TC (or FTC) +NVP** **Alternate first-line**: **AZT+ 3TC+EFV or TDF+3TC (or FTC) +EFV**	AZT+3TC+LPV/r or AZT+3TC+ABC	**Twice-daily AZT for 4-6w**, or **Once-daily NVP for 4-6w**

Year	Guidelines for initiation	Mother			Infant
		When to initiate	Recommended First-line regimen(s)	Recommended Second-line regimen(s)	Recommended prophylaxis started at birth
2012/ 2013	**Lifelong cART**, or **at least until after the cessation of breastfeeding**, regardless of WHO stage or CD4 cell count	**When to initiate**	**Recommended First-line regimen(s)**	**Recommended Second-line regimen(s)**	**Recommended prophylaxis started at birth**
2017	**Lifelong cART**	**As soon as HIV diagnosed**	**TDF+ FTC+EFV**	**TDF+ FTC**: with LPV/r, ATZ/r, DRV/r, RPV, or RAL **ABC+3TC**: with LPV/r, ATZ/r, DRV/r, RPV, or RAL Alternate second line: **AZT+3TC**: with LPV/r, ATZ/r, or DRV/r	**Twice-daily AZT for 6w**, or **Once-daily NVP for 6w**

Recommendations obtained from WHO guidelines (for corresponding years) for treating pregnant women LWH and prevention of HIV infection in infants [172–178].

Canadian guidelines strongly advocate cART treatment for all pregnant women LWH, irrespective of CD4 cell counts and HIV plasma viral load (pVL). Most recent guidelines recommend a combination of 2 NRTIs and a ritonavir-boosted PI (PI/r), due to proven safety and efficacy [179]. The non-boosted PI NFV was once used extensively in pregnancy. However, manufacturing issues between March and May 2007 led to a global recall of the drug [180], and it is no longer used. In addition to this, EFV and NVP were not recommended for use in pregnancy, owing to problems associated with potential teratogenicity and treatment complications, respectively [179]. More recently, the once daily, single-tablet regimen of ABC+3TC+DTG is increasingly used in pregnancy. Post-natal infant prophylaxis usually consisted of six weeks AZT, started within 6-12 hours after birth [179].

The number of pregnancies per year among women LWH in Canada has increased more than four times, from less than 50 per year in the early 1990s to ~230 in recent years (Figure 1.3). Treatment with cART in pregnancy has led to a dramatic decrease in MTCT rates in Canada. Between 1990 and 2010, the rate of MTCT was only 0.4% when cART was initiated at least four weeks before delivery [6]. With the successful implementation of immediate, lifelong cART at HIV diagnosis, the proportion of women conceiving while on cART is increasing steadily, reaching more than 60% in 2016. Figures 1.4 and 1.5 illustrate the timing of ART initiation and the composition of cART regimens during pregnancy respectively, for women LWH in Canada between 1990 and 2016.

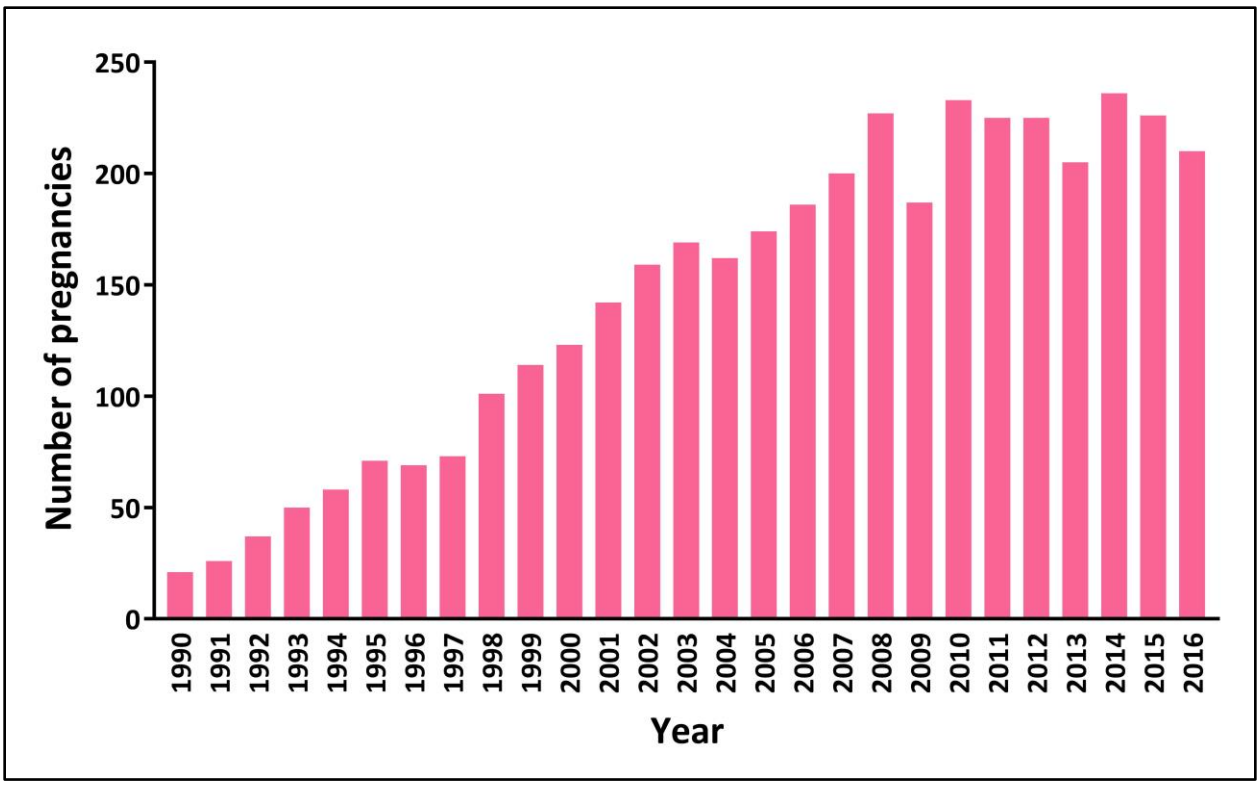

Figure 1.3. Number of pregnancies per year among women LWH in Canada between years 1990 and 2016. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email.

*Acknowledgements: CPHSP, Canadian HIV Trials Network (CTN) {analysis} and Public Health Agency of Canada (PHAC) {funding}.

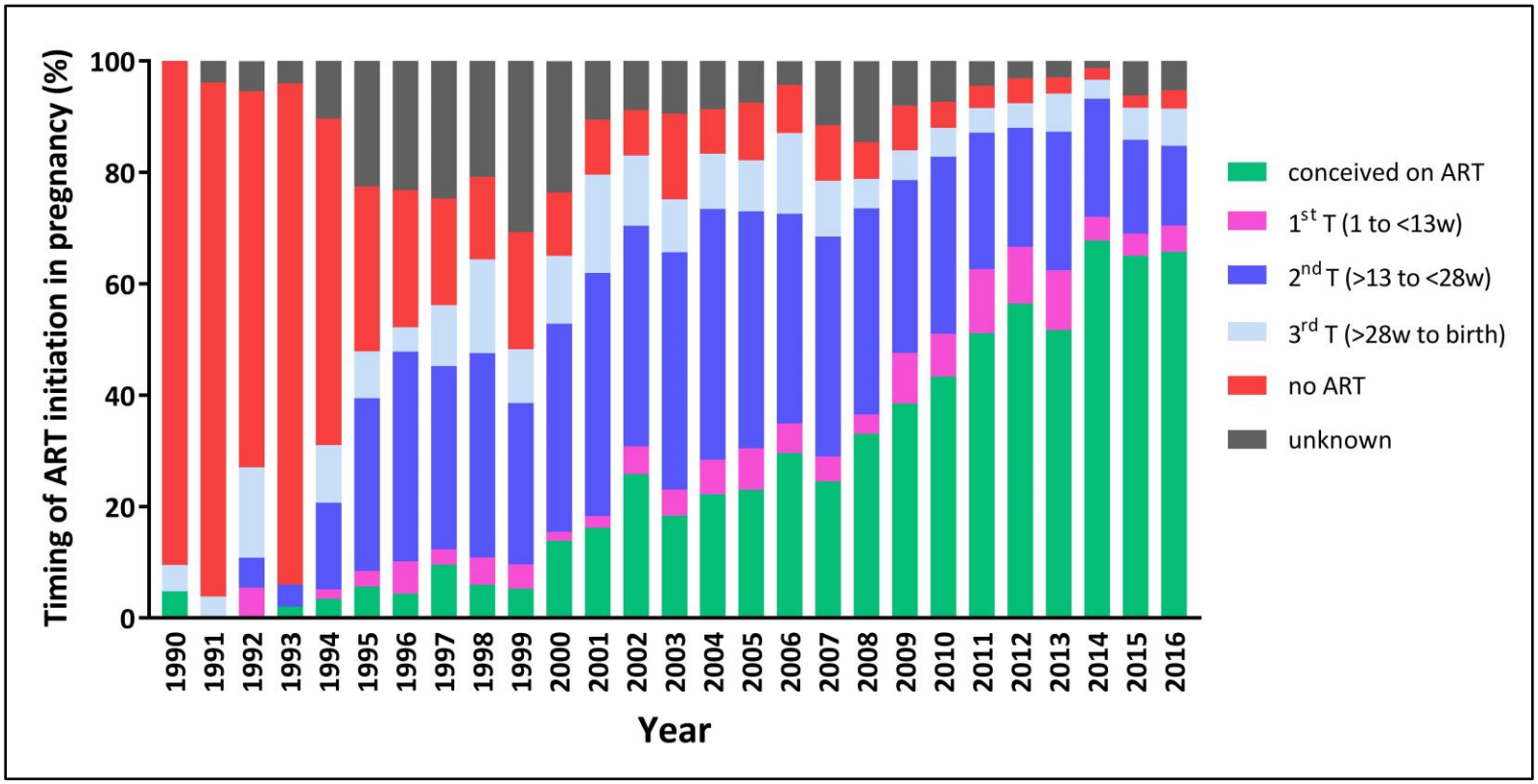

Figure 1.4. Timing of ART initiation in pregnancy for women LWH in Canada between 1990 and 2016. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email. *Acknowledgements: CPHSP, Canadian HIV Trials Network (CTN) {analysis} and Public Health Agency of Canada (PHAC) {funding}.

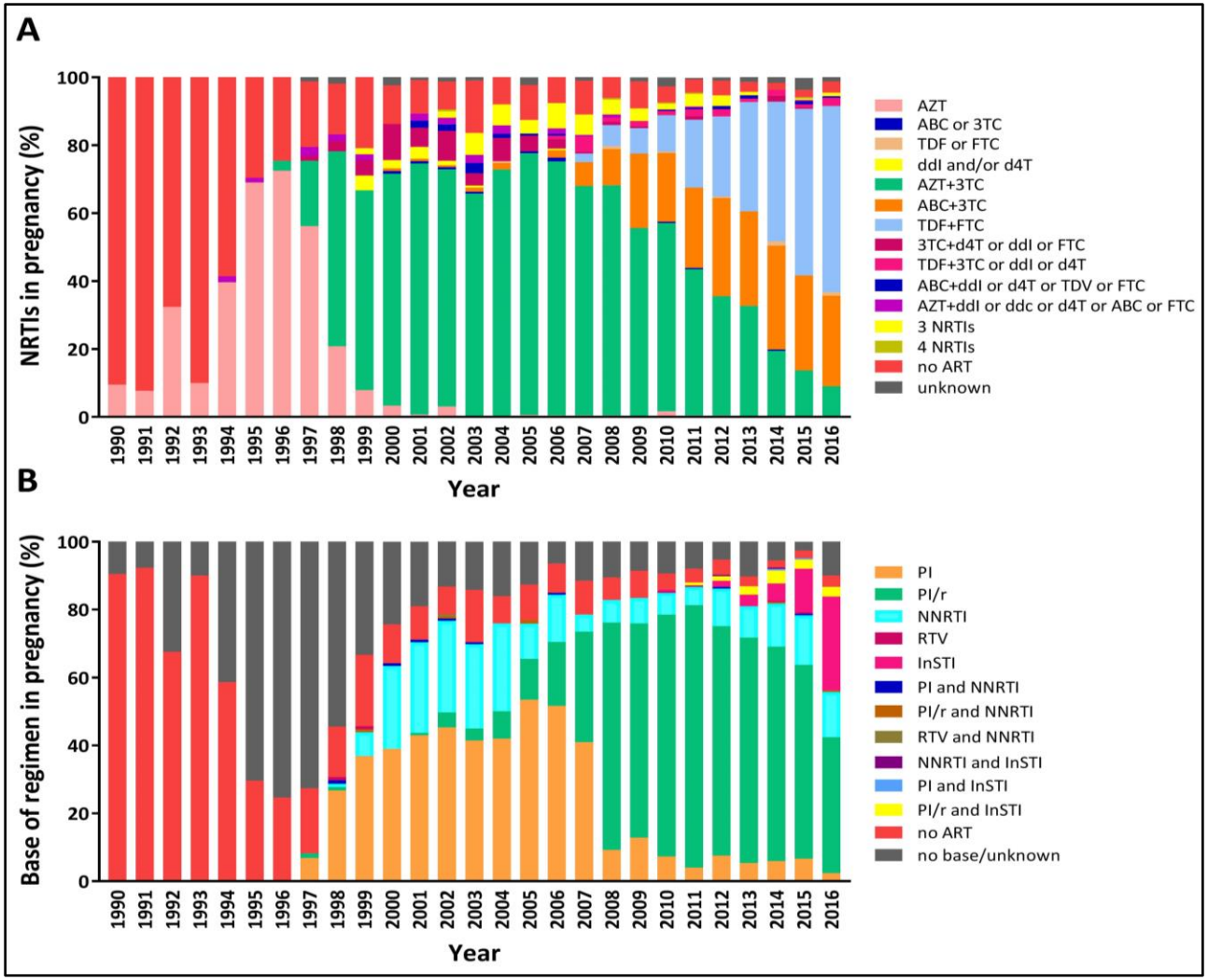

Figure 1.5. Composition of cART regimens received in pregnancy by women LWH in Canada between 1990 and 2016. (A) NRTIs used in pregnancy, and (B) Bases used in regimens. This figure was made using data obtained from the Canadian Perinatal HIV Surveillance Program (CPHSP). Permission to use data and figure was granted via email. *Acknowledgements: CPHSP, Canadian HIV Trials Network (CTN) {analysis} and Public Health Agency of Canada (PHAC) {funding}.

1.4.2 Outcomes in HIV/cART pregnancies

Although the benefits of cART during pregnancy in preventing MTCT are unquestionable and clearly outweigh the risks, a growing body of research has emerged suggesting adverse pregnancy outcomes among HIV-infected women. Several reports have demonstrated increased risks of preeclampsia, gestational diabetes, stillbirths, preterm birth, small for gestational age (SGA), low birth weight and intrauterine growth restriction in HIV pregnancies [181–188]. Comparatively, a few studies report no differences between HIV-infected and HIV-uninfected control women [189–191]. Some of these outcomes have been linked to specific ARVs; however, these associations are debated.

For instance, several studies report an increased risk of preterm birth with prenatal exposure to PI-based regimens [182,192–199], however others report no such association [200–203]. A few studies have further reported increased risk of preterm birth in association with the use of boosted-PIs compared to non-boosted PIs [193,194]. The use of PIs has also been associated with gestational diabetes[181]. With regards to other ARVs, a higher risk for low birth weight was detected among infants exposed to AZT-containing regimens *vs.* non-AZT regimens [204], and increased preterm birth, low birth weight and stillbirths have been reported among women receiving EFV-based cART compared to NVP-based regimens [205]. Of note, EFV is associated with neuropsychiatric symptoms in adults, including post-partum depression, sadness, mood changes and irritability [206]. Newer InSTIs such as RAL and DTG in pregnancy have been shown to be relatively safe with respect to adverse outcomes such as preterm birth, low birth weight, and SGA [207,208].

1.4.3 Outcomes in HIV-infected and HEU children

1.4.3.1 HIV-infected children

Treatment of perinatally HIV-infected children and adolescents with ARVs has considerably reduced mortality. However, as seen in adults, several ARV-associated metabolic complications have been reported in this population, including lipodystrophy syndrome, dyslipidemias, hyperlactatemia, bone and renal toxicities, cardiovascular and neuropsychological conditions, increased inflammation, and risk of cancer [209–213]. Many of these pathologies could be at least partially mediated by mitochondrial toxicity/dysfunction, which can also contribute to premature aging and immune senescence.

Consistent with this, decreased mtDNA content, reduced mitochondrial respiratory chain enzyme activity, and increased ROS have been reported among ART-treated children LWH, especially with the use of first-generation NRTIs such as d4T, ddI or AZT [127,214–218]. As seen in adults [111,128–130,132], mtDNA levels appear to increase following treatment interruption among children LWH [131]. However, this was accompanied by rapid decreases and increases in CD4 and CD8 cell counts, respectively, possibly due to increased cell turnover and immune activation in response to viral rebound [131,219]. Further, a recent study reported an accumulation of peripheral blood mononuclear cell (PBMC) mtDNA mutations with prolonged ARV treatment in children LWH exhibiting excellent virological response [220].

In addition to effects on mitochondria, Gianesin *et al.* reported significantly shorter PBMC TL and increased percentages of senescent, activated and exhausted CD8 T cells in children LWH, compared to both HEU and HIV-unexposed uninfected (HUU) controls [78],

suggesting accelerated cellular aging and immune senescence. Another group also reported shorter TL in both children LWH and HEU children compared to HUU controls[221]. Our group did not detect such differences in LTL between HIV groups [79], but this may be in part due to limitations regarding the age of the study participants which were not well balanced between groups. However, we did see ARV treatment having a positive effect on LTL [79].

1.4.3.2 HEU children

Aside from adverse outcomes at birth, it remains unclear whether *in utero* and/or postnatal ARV exposure may have long-term consequences on the health of HEU children. Many ARVs cross the placenta [37,38] and could affect cellular processes in the developing fetus.

Early findings from the PACTG 076 study were reassuring. There were no adverse effects related to weight, height, head circumference, cognitive/developmental function, malignancies, or increased death reported among HEU children with *in utero* and postnatal AZT exposure, after up to 5.6 years [222]. Since then however, increased risk of morbidity and mortality [223–226], stunted growth [227], musculoskeletal and cardiovascular congenital anomalies [228], immune system abnormalities [223,229,230], and inflammation [231,232] among HEU children with *in utero* cART exposure have been reported. ARV-associated hyperlactatemia, metabolic complications and neurodevelopmental, cognitive and language delays [233–240] have also been reported in young HEU children. Several of these conditions could implicate mitochondrial dysfunction. Zash *et al*. (2018) recently detected an early signal for increased incidence of neural-tube defects among Botswana children exposed to maternal DTG-containing regimen since conception [35]. Another group reported an association between

in utero tenofovir exposure and increased risk for speech impairment at three years of age among HEU children [238]. It should be noted however that other studies failed to detect either neurodevelopmental differences between ARV-exposed HEU and HUU [241], or any association between neurodevelopment and the type of ARV exposure among HEUs [237,242,243].

In relation to biomarkers of cellular aging, current literature is fairly inconsistent regarding the effects of *in utero* HIV/cART exposure on HEU TL and mtDNA. A few cross-sectional studies reported similar LTL among HEU and HUU children at birth [244,245] or later in life [78,79], while others observed shorter LTL [221] or cord blood TL [245] in HEU compared to HUU. Similarly, some cross-sectional studies reported lower mtDNA levels in HEU children [244,246–250], but others reported increased mtDNA content in HEU children at birth and during early life [251–253]. Our group recently reported increased blood mtDNA content in older HEU children diagnosed with autism spectrum disorder [254]. It should be noted that many of these studies were limited by sample size and/or unknown/unmatched characteristics of their control groups. The type of ARV exposure, for example older *vs.* newer NRTIs, may also explain some of these discrepancies. Given the ever-expanding global HEU population, together with the fact that a large majority of these individuals are now within their reproductive ages, further studies are needed to characterize the aging process in HEU populations, and evaluate the comparative safety of cART regimens used in pregnancy, in relation to their effects on markers of aging such as telomeres and mtDNA, and long-term HEU health.

Table 1.3 and 1.4 summarizes the literature on TL and blood mtDNA content in HIV+, HEU and HUU children

Table 1.3. Literature review of leukocyte or PBMC TL in HIV+, HEU and HUU children.

Study	HIV+ vs. HUU	HIV+ vs. HEU	HEU vs. HUU
Poirier MC *et al.* [244]	---	---	**No difference** in PBMC TL and cord blood LTL
Côté HCF *et al.* [79]	**No difference** in LTL Among HIV+, **children with detectable pVL** had **shorter LTL** than children with undetectable pVL	**No difference** in LTL	**No difference** in LTL
Imam T *et al.* [245]	---	---	**No difference** in LTL **Trend towards shorter cord Blood LTL** in HEU compared to HUU
Gianesin K *et al.* [78]	**Shorter** PBMC TL in HIV+ Among HIV+, **ART-naïve had shorter TL** than ART treated	**Shorter** PBMC TL in HIV+	**No difference** in PBMC TL
Shiau S *et al.* [221]	**Shorter** PBMC TL	**No difference** in LTL	**Shorter** PBMC TL in HEU

Table 1.4. Literature review of blood mtDNA content in HIV+, HEU and HUU children.

Study	HIV+ vs. HUU	HIV+ vs. HEU	HEU vs. HUU
Saitoh A *et al.* [218]	No control (HUU) group Among HIV+ children, those receiving **ddI had significantly reduced mtDNA levels** compared to children on non-ddI NRTI regimens, before the initiation of cART Following cART initiation, **children receiving ddI-containing cART had the least increase in mtDNA levels** compared to other NRTI-cART	---	---
Noguera-Julian A *et al.* [131]	No control (HUU) group MtDNA levels significantly **increased** among cART-treated HIV+ children who underwent one year planned treatment interruption	---	---
Moren C *et al.* [217]	Significant **reduction** in mtDNA levels	---	---
Moren C *et al.* [215]	Significant **mtDNA depletion** in HIV+ children with lipoatrophy/ lipohypertrophy	---	---

Study	HIV+ vs. HUU	HIV+ vs. HEU	HEU vs. HUU
Liu K *et al.* [214]	Significant mtDNA **depletion**	---	---
Poirier MC *et al.* [244]	---	---	**Lower cord blood and PBMC** mtDNA content Among HEUs, those **exposed *in utero* to maternal AZT had lower mtDNA content** compared to children without AZT exposure
Divi RL *et al.* [246]	---	---	Significant cord blood mtDNA **depletion**
Aldrovandi GM *et al.* [247]	---	---	Significantly **lower** PBMC mtDNA content Among HEUs, **mtDNA content was lowest** in children born to **ART-naïve mothers**, higher in HEUs exposed to AZT *in utero,* and higher still in children with combination NRTI exposure *in utero.*
Brogly SB *et al.* [250]	---	---	cART-exposed HEU children with and without mitochondrial dysfunction had significantly **lower** mtDNA levels than HUU
Hernandez S *et al.* [248]	---	---	Significantly **lower** cord blood PBMC mtDNA content

Study	HIV+ vs. HUU	HIV+ vs. HEU	HEU vs. HUU
Jao J *et al.* [249]	---	---	*In utero* cART-exposed HEU children with 6w AZT prophylaxis had significantly **lower** mtDNA content compared to HUU HEU with NVP prophylaxis had similar mtDNA content as HUU children
Côté HCF *et al.* [255]	---	---	cART-exposed HEU children tended to have **higher** mtDNA content at birth compared to HUU HEU mtDNA content increased during AZT prophylaxis period (6w) and **remained significantly higher** than HUU at all subsequent time points (up to 8 months).
McComsey GA *et al.* [252]	---	---	Significantly **elevated** PBMC mtDNA content at birth
Ross AC *et al.* [253]	---	---	Significantly **elevated** PBMC mtDNA content at birth
Budd MA *et al.* [254]	---	---	HEU children with and without autism spectrum disorder had significantly **higher** blood mtDNA content than HUU children without autism spectrum disorder

1.5 Research Objective and Hypothesis

Alterations in TL and mtDNA levels at birth, and/or early in life, may reflect accelerated aging and exert long-term health effects on HEU children in the absence of clinical symptoms. There is an urgent need to investigate such subclinical alterations, in order to optimize the safety of cART regimens used during pregnancy. However, because we did not study cART-unexposed HEUs, it is challenging to distinguish between the effects of exposure to maternal HIV milieu *vs.* those of *in utero* exposure to cART. The overall goal of my PhD research was to investigate the effects of cART on biomarkers of cellular and mitochondrial health, in both clinical samples and cell culture models. The overarching hypothesis of my research was that both LTL and mtDNA content would be affected by cART. My objectives were to:

1) Compare LTL and blood mtDNA at birth, and over the first three years of life, among HEU and HUU children, and investigate relationships with *in utero* cART exposure.
2) Investigate changes in mitochondrial health (mass, MMP, ROS), mtDNA content, and cellular apoptosis following exposure to new/clinically relevant cART regimens at pharmacological concentrations, and explore the role of hTERT in protecting against cART-induced mitochondrial damage (if any), using a telomerase-independent transformed cell line.

Chapter 2: LEUKOCYTE TELOMERE LENGTH AT BIRTH AND DURING THE EARLY LIFE OF CHILDREN EXPOSED TO BUT UNINFECTED WITH HIV AFTER *IN UTERO* EXPOSURE TO ANTIRETROVIRALS

2.1 Abbreviated introduction to chapter

The objective of this study was to compare LTL at birth in a large cohort of HEU and HUU children, and investigate LTL relationship with *in utero* exposure to maternal cART. Furthermore, I report for the first time the longitudinal dynamics of LTL over the first three years of life among HEU children.

2.2 Methods

2.2.1 Study population

Study participants were all HEU and HUU children ≤ 3 years of age. All HEU and half the HUU children were enrolled in three Canadian cohort studies: 1) a pediatric cohort (born between 2003 and 2006 in Vancouver, BC, or Toronto, ON) [255] which collected longitudinal whole blood (WB) specimens from HEUs between birth and 9 months, and single specimens from HUUs aged 0-9m; 2) a pregnancy cohort (born between 2005 and 2009 in Vancouver, BC) [245] which collected single WB specimens from HEUs between birth and 15 months, and from HUUs at birth only; and 3) the Children and Women: AntiRetrovirals and Markers of Aging (CARMA) cohort (born between 2006 and 2012 in Vancouver, BC, Toronto, ON, Ottawa, ON, or Montreal, QC) [79] which collects longitudinal WB specimens from HEUs between birth and

19 years, and single specimens from HUUs aged 0-19 years (Figure 2.1). The remaining HUU children were anonymous controls (all from Vancouver, BC) for whom only sex and date of birth were known. Their WB samples were leftovers from blood work during routine hospital visits or for reasons such as elective surgery. Inclusion/exclusion criteria for the three cohorts are described in Table 2.1. Of note, all participants with a birth specimen were from Vancouver, BC. This study was reviewed and approved by the Institutional Review Board of all participating institutions, and written informed consent was obtained from the parents/guardians of all study participants. The latter was not required for anonymous controls. Maternal demographic and clinical data, including exposures during pregnancy were extracted from the cohort databases. Maternal ethnicity was self-reported. Since paternal ethnicity was not always available, infant ethnicity was reported as that of the mother. Data on smoking during pregnancy was self-reported but collected with different levels of detail in each cohort. We therefore categorized smoking (tobacco) within this analysis as self-reported smoking at any time (ever) during pregnancy (yes *vs.* no), irrespective of the intensity, frequency, and duration of cigarette smoking. As many children were enrolled in early childhood during visits to their pediatrician, information relating to maternal clinical characteristics during pregnancy (e.g. HIV plasma viral load (pVL), co-infections, etc.) was not always available. A detectable pVL was defined as >50 copies/ml. Preterm delivery was defined as births occurring <37 weeks of gestation. Small for gestational age (SGA) was defined as a birth weight below the 10^{th} percentile relative to Canadian neonates of the same gestational age (GA) [256].

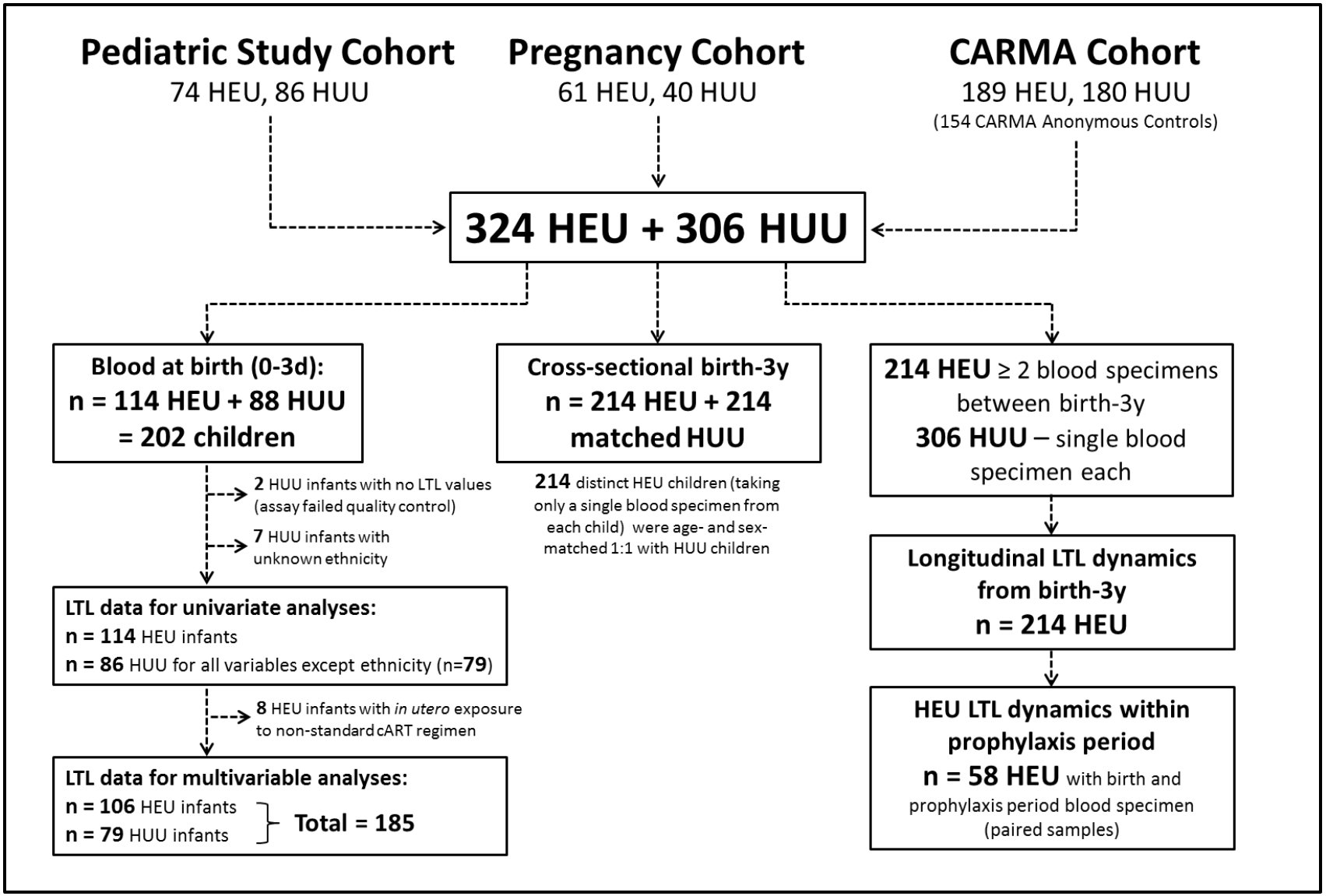

Figure 2.1. Schematic showing participant selection, as well as their inclusion in different analyses.

Table 2.1. Inclusion/exclusion criteria of the three study cohorts

	Inclusion criteria	Exclusion criteria
Pediatric Study Cohort	All HEU and HUU infants in this cohort were born between July 2003 and June 2006. **HEU** 1. Infants born to HIV-infected women who received cART during pregnancy and intravenous AZT during labor, and 2. Infants who received oral AZT prophylaxis during the first 6 weeks of life starting within ~12 hours of birth **HUU** 1. Infants born to HIV-uninfected mothers and enrolled from 3 sources: (i) infants (1-6 months old) having blood work done prior to elective minor pediatric surgery, (ii) neonates (0–3 days old) born at Children's and Women's Health Centre of British Columbia, and (iii) infants (1 day–8 months old) having blood work done for various minor medical reasons.	Controls were excluded if they were known to have a mitochondrial disorder or had a serious and/or febrile illness.
Pregnancy Cohort	Infants born to HIV-infected women and HIV-uninfected controls who were enrolled during pregnancy in a mother/child HIV cohort study at British Columbia Women's Hospital, Vancouver, Canada, from 2005 to 2009. All HIV-infected participants received cART, as per time-specific clinical guidelines.	None
CARMA Cohort	HEU and HUU children, aged 0-19 years, born to women enrolled in the prospective CARMA cohort at 4 sites: Vancouver, BC, Toronto, ON, Ottawa, ON, or Montreal, QC. Almost all HEU children were exposed to ART *in utero* and/or during post-natal prophylaxis. The majority of HUU children were anonymous controls for whom blood specimens were leftovers from blood work during hospital visits or for reasons such as minor elective surgeries.	None

2.2.2 Leukocyte telomere length measurement

WB relative LTL was measured via monochrome multiplex quantitative polymerase chain reaction (MMqPCR) as previously described [257]. Relative LTL was expressed as a ratio of the quantity of telomeric DNA (T) normalized to the copy number of a single-copy nuclear gene (S), yielding the T/S ratio [257]. Ratios were calibrated to approximate absolute TL (in kilobases) by applying a coefficient (measurement X 2.33), as previously described [257]. All study specimens were randomized and those collected from a given study participant were assayed simultaneously to avoid inter-run variability. Specimens that did not meet quality control criteria [257], namely <15% difference between replicates over two assay attempts, were excluded. The intra and inter-assay variability were 4.2% and 4.3%, respectively.

2.2.3 Statistical analyses

The χ^2, Student's t, and Mann-Whitney U tests were used to compare clinical and demographic characteristics of HEU and HUU study participants. These as well as Pearson's/Spearman's correlations were used to investigate univariate associations between LTL at birth and the following explanatory variables: HIV exposure status (HEU *vs.* HUU), infant sex, GA at birth, birth weight, SGA, maternal ethnicity, age at delivery, smoking ever during pregnancy, and duration and type of cART (AZT+3TC+LPV/r *vs.* AZT+3TC+NFV *vs.* AZT+3TC+NVP *vs.* ABC+3TC+PI/r *vs.* TDF+FTC or 3TC+PI/r) during pregnancy. Multiple group comparisons were done using Kruskal-Wallis' test followed by Dunn's multiple pairwise comparison test if indicated. Apart from duration and type of cART which were forced in as per an *a priori* decision, factors found to be important univariately ($p<0.15$) were all considered while developing multivariable ANCOVAs using backward stepwise selection. Equality of

variances for two or more groups was verified using Levene's test. Interactions between HEU/HUU status and other variables of interest were also examined and, if present, were included in the model. For cross-sectional comparison of LTL during the first three years of life, a subset of HEU and HUU children were sex- and age-matched 1:1 (± 2 days within the first 2 weeks; ± 8 days from 2 weeks to 1 year; ± 15 days from 1-3 years). The relationship between LTL and age was then evaluated by comparing the linear regression's slopes of the two groups using GraphPad Prism v7. Analysis of HEU LTL dynamics between birth and the closest subsequent visit during the prophylaxis period was performed using the Wilcoxon signed-rank paired test. Finally, to assess the relationship between age and LTL, longitudinal LTL dynamics in HEU children was analyzed using a generalized mixed effects additive model with the 'mgcv' package in R.

2.3 Results

2.3.1 Characteristics of study participants

2.3.1.1 All participants

WB specimens were available for 324 HEU and 306 HUU children. Of these, 214 HEU children (66%) had ≥ 2 blood specimens collected between birth and three years of age whereas all HUU children had only a single blood specimen each (Figure 2.1). Characteristics of all the children (and their mothers) are presented in Table 2.2(A). Demographic information was unavailable for 154 anonymous HUUs. For the remainder, HEU and HUU children were very similar, apart from their ethnicity whereby, based on their mother's self-reported ethnicity ~50% of HEUs but less than 1% of HUUs were Black/African Canadian. Rates of maternal smoking during pregnancy, although high, were comparable between groups, with 33% of HIV+ and 30% of HIV- mothers self-reporting to have ever smoked during their pregnancy. With respect to *in utero* cART exposure, the most common regimen backbone was AZT+3TC. Approximately a third of HEUs (n=112, 35%) were exposed to maternal AZT+3TC+LPV/r, 83 (26%) to AZT+3TC+NFV, 26 (8%) to AZT+3TC+NVP, 32 (10%) to ABC+3TC+PI/r and 27 (8%) to TDF+FTC or 3TC+PI/r. The remaining 36 HEUs (11%) were exposed to other non-standard cART regimen (detailed in Table 2.3). Seven (2%) HEU children were born to ART naïve HIV+ mothers.

Table 2.2. Demographic and clinical characteristics of HEU and HUU children: (A) all participants (B) children with a blood specimen at birth

	(A) All participants			(B) Children with birth specimen		
	HEU (n = 324)	**HUU (n = 306)**	**p value**	**HEU (n = 114)**	**HUU (n = 88)[a]**	**p value**
Infant Characteristics						
Male sex	161 (50)	169 (55)	0.16	64 (56)	47 (53)	0.70
GA, weeks	38.4 (27.1 - 41.7) (n=323)	39.0 (28.3 - 42.1) (n=149)	0.07	38.3 (31.3 - 41.6)	39.4 (28.9 - 42.1)	**< 0.001**
Preterm delivery (<37 weeks)	61 (19)	28 (19)	0.98	23 (20)	9 (10)	0.055
< 37 and ≥ 34 weeks	42 (13)	17 (11)		19 (17)	8 (9)	
< 34 weeks	19 (6)	11 (7)		4 (4)	1 (1)	
Birth weight, kg	3.1 (1.6 – 4.2) (n=184)	3.2 (1.2 - 5.2) (n=150)	0.32	3.1 (1.6 - 4.1)	3.4 (1.4 - 5.2)	**< 0.001**
SGA	30 (16) (n=184)	27 (18) (n=149)	0.66	19 (17)	12 (14)	0.55
Maternal Characteristics						
Maternal age at delivery, years	31.5 (16.7 - 45.3)	32.2 (16.7 - 44.0) (n=135)	0.15	31.0 (17.4 - 42.4)	32.5 (21.3 - 43.0)	0.063
Maternal ethnicity			**< 0.001**			**< 0.001**
Indigenous	42 (13)	9 (3)		33 (29)	7 (8)	
Black/African Canadian	158 (49)	2 (0.7)		28 (25)	2 (2)	
White	84 (26)	93 (30)		39 (34)	55 (63)	
Asian	21 (6)	29 (9)		10 (9)	16 (18)	
Other/Unknown	12/7 (6)	19/154 (57)		4/0 (4)	1/7 (9)	
Maternal smoking ever in pregnancy	105 (33) (n=322)	45 (30) (n=152)	0.51	63 (55)	38 (43)	0.09
Detectable HIV pVL (>50 copies/ml) close to delivery	18 (12) (n=145)	NA		12 (12) (n=99)	NA	
Indigenous	42 (13)	9 (3)		33 (29)	7 (8)	
Black/African Canadian	158 (49)	2 (0.7)		28 (25)	2 (2)	

	(A) All participants			(B) Children with birth specimen		
	HEU (n = 324)	**HUU (n = 306)**	**p value**	**HEU (n = 114)**	**HUU (n = 88)[a]**	**p value**
White	84 (26)	93 (30)		39 (34)	55 (63)	
Asian	21 (6)	29 (9)		10 (9)	16 (18)	
Other/Unknown	12/7 (6)	19/154 (57)		4/0 (4)	1/7 (9)	
cART Characteristics						
Duration of *in utero* cART exposure, weeks	24.4 (0.0 - 41.7) (n=322)	NA		20.6 (0.0 - 41.1)	NA	
Maternal cART initiation						
Before conception	124 (38)	NA		40 (35)	NA	
In 1st trimester	39 (12)	NA		15 (13)	NA	
In 2nd trimester	124 (38)	NA		46 (40)	NA	
In 3rd trimester	29 (9)	NA		13 (11)	NA	
Naïve/unknown	7/1 (2)	NA		0 (0)	NA	
Maternal cART regimen						
AZT+3TC+NVP	26 (8)	NA		7 (6)	NA	
AZT+3TC+NFV	83 (26)	NA		34 (30)	NA	
AZT+3TC+LPV/r	112 (35)	NA		45 (39)	NA	
ABC+3TC+PI/r	32 (10)	NA		10 (9)	NA	
TDF+FTC(or 3TC)+PI/r	27 (8)	NA		10 (9)	NA	
Other[b]	36 (11)	NA		8 (7)	NA	
Naïve/Unknown	7/1 (2)	NA		0 (0)	NA	
Infant AZT prophylaxis, weeks	6 (0 - 8) (n=323)[c]	NA		5 (2 - 6)	NA	

Data are presented as n (%) or median (range). Abbreviations: HEU – HIV-exposed uninfected, HUU – HIV-unexposed uninfected, GA – Gestational Age, SGA – Small for Gestational Age, pVL – plasma Viral Load, cART – combination Antiretroviral Therapy, AZT – Zidovudine, 3TC – Lamivudine, NVP – Nevirapine, NFV – Nelfinavir, LPV/r – Ritonavir-boosted Lopinavir, ABC – Abacavir, PI/r – Ritonavir-boosted Protease Inhibitor, TDF – Tenofovir Disoproxil Fumarate, FTC - Emtricitabine

Twenty-nine sets of siblings (25 HEU and 4 HUU) were identified among all study participants, of which five were HEU twin pairs.

[a]Three anonymous HUU controls (with no information apart from age and sex) were not listed in this Table as they could not be included in multivariable models.

[b]List of "Other" cART regimen is detailed in Table 2.3.

[c]93 HEU children received AZT+3TC postnatal prophylaxis

Table 2.3. Breakdown of the "Other" maternal cART regimens during pregnancy.

Regimen	(A) All HEUs (n = 36)	(B) HEUs with birth specimen (n = 8)
ABC+3TC	2 (6)	---
ABC+3TC+d4T	1 (3)	---
ABC+3TC+ddI	1 (3)	---
ABC+3TC+NVP	3 (8)	1 (13)
ABC+d4T+LPV/r	1 (3)	---
ABC+TDF+ATV/r	1 (3)	---
AZT+3TC	2 (6)	---
AZT+3TC+ABC	5 (14)	1 (13)
AZT+3TC+ddI+NFV	1 (3)	---
AZT+3TC+EFV	1 (3)	1 (13)
AZT+3TC+TDF+PI/r	2 (6)	---
AZT+ABC+NFV	1 (3)	---
AZT+ABC+NVP	1 (3)	---
AZT+ddI+NFV	1 (3)	---
d4T+3TC+PI/r	3 (8)	1 (13)
d4T+3TC+NFV	2 (6)	1 (13)
d4T+3TC+NVP	1 (3)	1 (13)
ddI+3TC+NFV	1 (3)	---
ddI+3TC+NVP	1 (3)	---
RAL+ETV+DRV/r	1 (3)	1 (13)
TDF+ddI+SAQ/r	1 (3)	---
TDF+FTC+d4T+RTV	1 (3)	---
TDF+FTC+EFV	1 (3)	---
TDF+NVP+LPV/r	1 (3)	1 (13)

2.3.1.2 Participants with a birth specimen

A birth (0-3d) specimen and demographic information were available for 114 HEU and 88 HUU children. Their characteristics are shown in Table 2.2(B). The majority of these HEU infants were of Indigenous (29%) and Black/African Canadian (25%) ethnicity, while 63% of HUU infants were White. There was no difference in infant sex or SGA between the groups. However, HEU infants had lower GA and weight at birth (both $p<0.001$) and tended toward a higher risk of preterm birth ($p=0.055$). Within these 202 participants, rates of maternal smoking during pregnancy were higher ($p<0.001$) than the overall group, with 55% of HIV+ and 43% of HIV- mothers self-reporting having ever smoked during their pregnancy. Detailed smoking intensity data was available for a subset of mothers (HIV+ n=44; HIV- n=11), and was similar between the two groups (median [interquartile range, IQR] cigarettes/day – HIV+ women: 10.0 [5.0-10.5] *vs.* HIV- women: 5.0 [3.5-11], $p=0.48$). All pregnant women received cART during their pregnancy, and once again, AZT+3TC+LPV/r and AZT+3TC+NFV were the most common regimens. At the visit closest to delivery, 88% of women had undetectable pVL.

2.3.2 Infant LTL at birth

LTL data was obtained for all infants, but for two HUUs measurements did not pass assay quality control (Figure 2.1). In univariate analyses, LTL at birth was similar between HEU and HUU infants (Figure 2.2A, Table 2.4). Longer LTL was associated with female sex in the combined group as well as in the HEU and HUU subgroups (Figure 2.2B, Table 2.4). Lower infant birth weight was significantly associated with longer LTL at birth in the combined group and in the HEU subgroup (Table 2.4). Being SGA was associated with longer LTL only in the HEU subgroup (Table 2.4). Additionally, there was an association between LTL and ethnicity

(Kruskal Wallis p=0.045), whereby infants born to Black/African Canadian mothers had significantly longer birth LTL compared to infants born to Indigenous mothers but not White mothers (Figure 2.3). Of note, a significant interaction (p=0.009) between HEU/HUU status and maternal smoking ever during pregnancy was observed, with the latter being associated with longer LTL in HUUs but shorter LTL in HEUs (Figure 2.2C).

Among HEU infants, neither duration of cART exposure *in utero* nor maternal pVL status (detectable vs. undetectable) close to delivery were related to HEU birth LTL. However, HEUs exposed to AZT+3TC+NVP during pregnancy had significantly longer birth LTL compared to HEUs exposed to AZT+3TC+LPV/r (p=0.01) or HUU controls (p=0.034, Figure 2.2D).

Table 2.4. Univariate analyses of the association between possible predictors and infant leukocyte telomere length (LTL) at birth

Explanatory variables	All infants (n = 200)		HEUs (n = 114)		HUUs (n = 86)	
	r_s[a]	p value	r_s	p value	r_s	p value
HIV exposure status (HEU vs. HUU)	---	0.43	---	---	---	---
Infant Sex, Male (ref. Female)	---	**0.003**	---	**0.04**	---	**0.03**
GA (per week)	-0.115	0.10	-0.033	0.73	-0.158	0.15
Preterm Birth (ref. Term birth)	---	0.17	---	0.86	---	0.06
Birth Weight (per kg)	-0.176	**0.01**	-0.244	**0.009**	-0.037	0.74
SGA, Yes (ref. No)	---	0.11	---	**0.02**	---	0.79
Maternal age (per year)	-0.009	0.90	0.088	0.35	-0.115	0.29
Maternal ethnicity:						
Indigenous	---	**0.045**[b]	---	**0.007**[b]	---	0.79[b]
Black/African Canadian						
White						
Asian and Others						
Maternal smoking ever in pregnancy, Yes (ref. No)	---	0.82	---	**0.005**	---	**0.002**
HIV pVL (>50 copies/ml) close to delivery, Detectable (ref. undetectable)	---	----	---	0.76 (n = 84)	---	---

Explanatory variables	All infants (n = 200)		HEUs (n = 114)		HUUs (n = 86)	
	r_s[a]	p value	r_s	p value	r_s	p value
Duration of *in utero* cART exposure (per week)	---	---	0.019	0.84	---	---
Maternal cART started:						
Before conception	---	---	---	0.61[b]	---	---
1st trimester						
2nd trimester						
3rd trimester						
Maternal cART regimen:						
AZT+3TC+NVP	---	---	---	0.21[b]	---	---
AZT+3TC+NFV						
AZT+3TC+LPV/r						
ABC+3TC+PI/r						
TDF+FTC(or 3TC)+PI/r						

r_s[a] - Spearman's rho, [b]Kruskal-Wallis p value

Abbreviations: HEU – HIV-exposed uninfected, HUU – HIV-unexposed uninfected, GA – Gestational Age, SGA – Small for Gestational Age, cART – combination Antiretroviral Therapy, AZT – Zidovudine, 3TC – Lamivudine, NVP – Nevirapine, NFV – Nelfinavir, LPVr – Ritonavir-boosted Lopinavir, ABC – Abacavir, PI/r – Ritonavir-boosted Protease Inhibitor, TDF – Tenofovir Disoproxil Fumarate, FTC – Emtricitabine

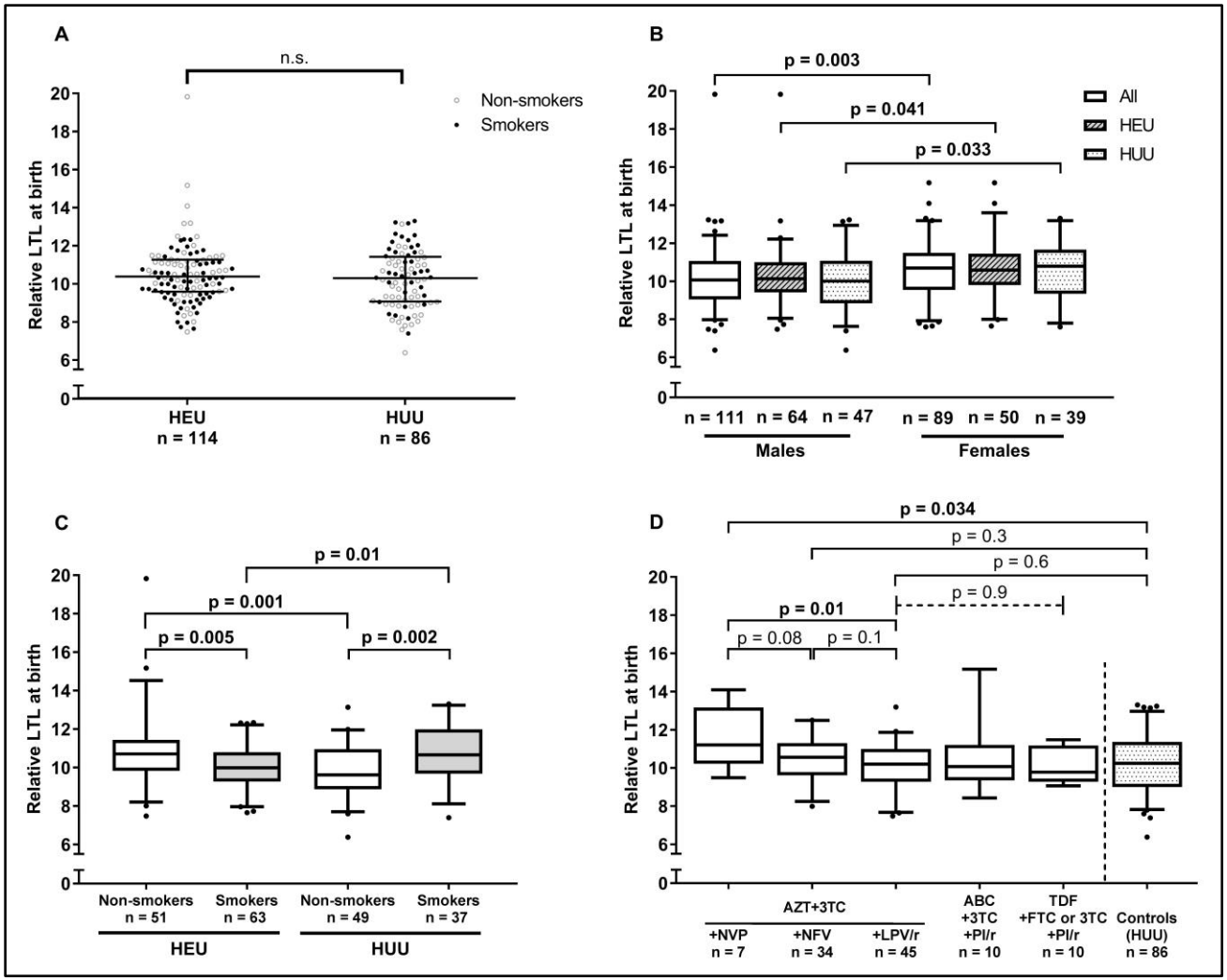

Figure 2.2. Unadjusted comparisons of LTL at birth between **A** – HEU and HUU infants (Mann-Whitney U test). **B** – males and females (Mann-Whitney U test). **C** –HEUs and HUUs exposed to maternal smoking during pregnancy or not (Mann-Whitney U test). **D** – HEUs exposed to different cART regimen in utero and HUUs [solid brackets indicate Student's t-test (ANOVA 3-group comparison of AZT+3TC backbone regimen, p=0.02) whereas the dashed bracket indicates Kruskal Wallis p value]. For panels B-D, whiskers of the box plots represent the 5-95 percentiles.

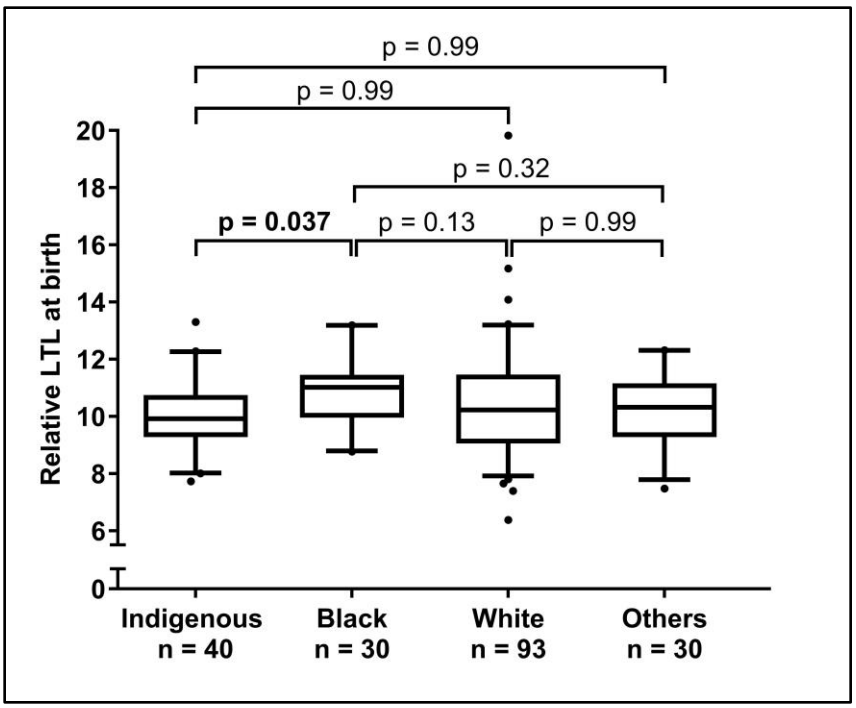

Figure 2.3. Unadjusted comparisons of LTL at birth between Indigenous, Black/African Canadian, White and Other ethnicities (all participants; Kruskal Wallis p=0.045, individual p values were obtained by Dunn's multiple pairwise comparison test). LTL measurements of one participant from each of the "White" and "other" groups did not pass assay quality control.

In a multivariable model of children with a birth specimen (n=185, Figure 2.4A) that included HEU/HUU status, infant sex, birth weight, ethnicity, maternal smoking ever during pregnancy and the HEU/HUU status*maternal smoking interaction term, female sex remained independently associated with longer infant LTL at birth. With respect to the other variables, although the model suggests that HEU status and being born to mothers who smoked during pregnancy were both independently associated with longer LTL at birth, this must be interpreted with caution given the behaviour of the interaction term. The latter clearly indicates that HEU infants born to mothers who smoked during pregnancy had significantly shorter LTL at birth ($p<0.001$) compared to HUU infants born to mothers who did not smoke during pregnancy, indicating that the direction of the effect of maternal smoking is opposite in the two groups (Figure 2.4C-D). In a similar model that separated HEU children according to the type of *in utero* cART exposure (Figure 2.4B), HEUs exposed to AZT+3TC+NVP and AZT+3TC+NFV had significantly longer birth LTL compared to HUU children. Additionally, although HIV+ mothers tended to be younger than HIV- mothers ($p=0.063$), the results did not change when maternal age at delivery was forced into the models (data not shown).

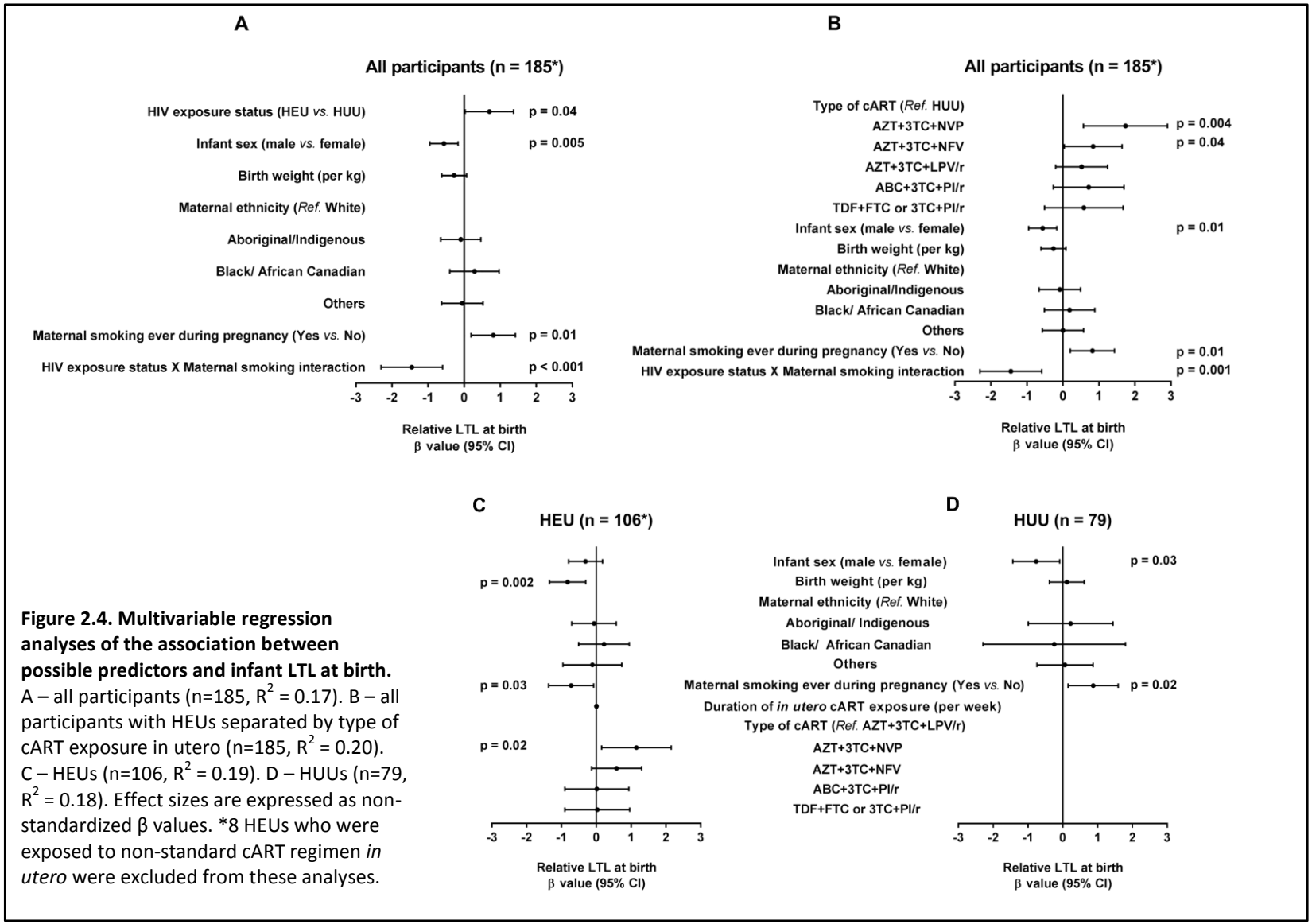

Figure 2.4. Multivariable regression analyses of the association between possible predictors and infant LTL at birth. A – all participants (n=185, R^2 = 0.17). B – all participants with HEUs separated by type of cART exposure in utero (n=185, R^2 = 0.20). C – HEUs (n=106, R^2 = 0.19). D – HUUs (n=79, R^2 = 0.18). Effect sizes are expressed as non-standardized β values. *8 HEUs who were exposed to non-standard cART regimen *in utero* were excluded from these analyses.

In the multivariable model restricted to the HEU group (n=106, Figure 2.4C), lower birth weight, being born to mothers who never smoked during pregnancy and being exposed to AZT+3TC+NVP (compared to AZT+3TC+LPV/r) remained independently associated with longer HEU LTL at birth, while duration of cART exposure continued to show no association. In the final multivariable model of HUUs, restricted to the 79 children who had known ethnicity (Figure 2.4D), female sex and being born to mothers who smoked during pregnancy, but not lower birth weight, were independently associated with longer LTL at birth.

Given an imbalance in ethnicity between the groups, whereby HEU infants were more likely to be of Indigenous or Black/African Canadian ethnicity compared to HUU infants (53% *vs.* 10%), a sensitivity analysis that included only children born to White mothers (n=93; 39 HEU, 54 HUU) was performed. The results were essentially unchanged compared to that of the full sample (Figure 2.5), suggesting that the associations observed with LTL at birth were not confounded by ethnicity *per se*.

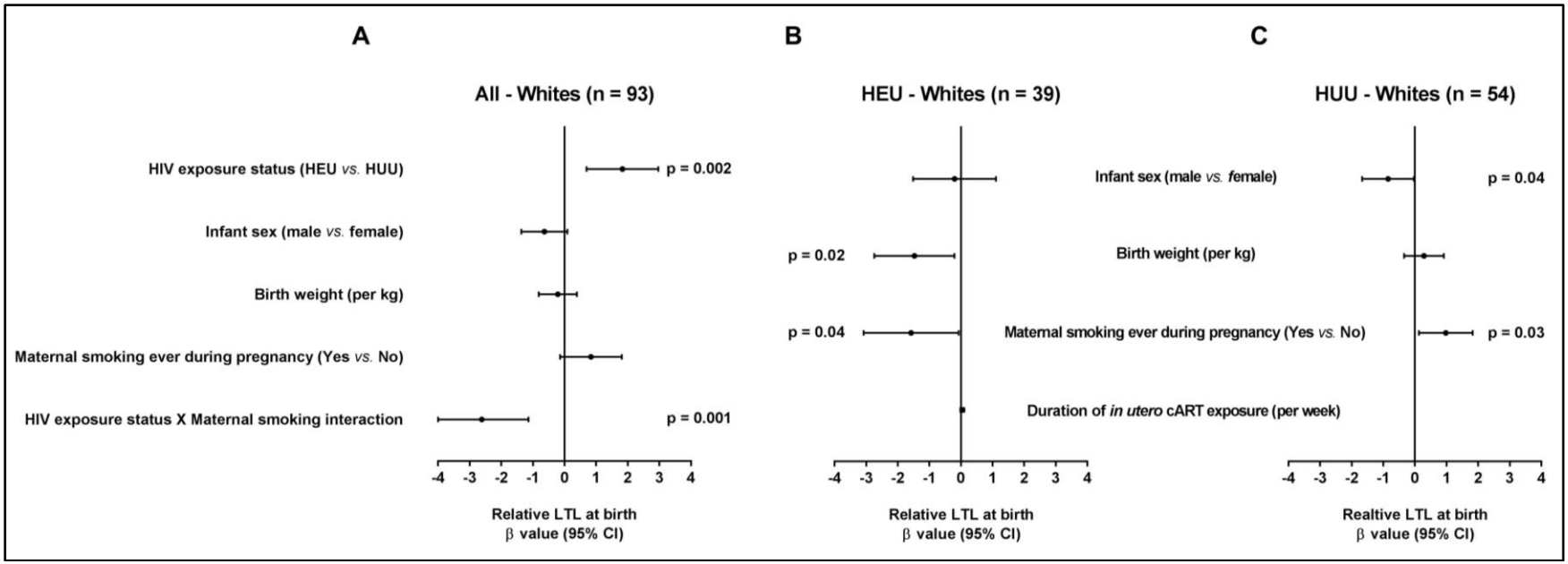

Figure 2.5. Multivariable regression analyses among White participants (sensitivity analysis) showing the association between possible predictors and infant LTL at birth. A – all (n=93, R^2 = 0.18). B – HEUs (n=39, R^2 = 0.30). C – HUUs (n=54, R^2 = 0.20). Effect sizes are expressed as non-standardized β values.

2.3.3 Cross-sectional comparison of group LTL during the first three years of life

For a total of 214 distinct HEU children aged 0-3 years, a single specimen was matched 1:1 with those of HUU children of same age and sex. Their characteristics (both infant and maternal) are detailed in Table 2.5.

Table 2.5. Demographic and clinical characteristics of age- and sex-matched HEU and HUU children

	HEU (n = 214)	HUU (n = 214)	p value
Infant Characteristics			
Male sex	112 (52)	112 (52)	1.00
GA, weeks	38.4 (27.1 - 41.6)	39.1 (28.9 - 42.1) (n=99)	**0.010**
Preterm delivery (<37 weeks)	43 (20)	18 (18)	0.69
< 37 and ≥ 34 weeks	32 (15)	11 (11)	
< 34 weeks	11 (5)	7 (7)	
Birth weight, kg	3.1 (1.6 - 4.2) (n=141)	3.4 (1.2 - 4.8) (n=100)	0.054
SGA	22 (16) (n=141)	18 (18) (n=99)	0.60
Maternal Characteristics			
Maternal age at delivery, years	31.3 (17.4 - 45.3)	32.2 (22.2 - 44.0) (n=87)	0.09
Maternal ethnicity			**< 0.001**
Indigenous	33 (15)	5 (2)	
Black/African Canadian	90 (42)	2 (1)	
White	62 (29)	55 (26)	
Asian	18 (9)	22 (10)	
Other/Unknown	7/4 (5)	5/125 (61)	
Maternal smoking ever in pregnancy	76 (36)	22 (22) (n=101)	**0.014**
Detectable HIV pVL (>50 copies/ml) close to delivery	14 (12) (n=113)	NA	

	HEU (n = 214)	HUU (n = 214)	p value
cART Characteristics			
Duration of *in utero* cART exposure, weeks	23.0 (0.0 - 41.1) (n=212)	NA	
Maternal cART initiation			
Before conception	82 (38)	NA	
In 1st trimester	23 (11)	NA	
In 2nd trimester	84 (39)	NA	
In 3rd trimester	20 (9)	NA	
Naïve/unknown	4/1 (2)	NA	
Maternal cART regimen			
AZT+3TC+NVP	16 (7)	NA	
AZT+3TC+NFV	55 (26)	NA	
AZT+3TC+LPV/r	80 (37)	NA	
ABC+3TC+PI/r	22 (10)	NA	
TDF+FTC(or 3TC)+PI/r	15 (7)	NA	
Other	21 (10)	NA	
Naïve/unknown	4/1 (2)	NA	
Infant AZT prophylaxis, weeks	6 (0-8)	NA	

Data are presented as n (%) or median (range). Abbreviations: HEU – HIV-exposed uninfected, HUU – HIV-unexposed uninfected, GA – Gestational Age, SGA – Small for Gestational Age, pVL – plasma Viral Load, cART – combination Antiretroviral Therapy, AZT – Zidovudine, 3TC – Lamivudine, NVP – Nevirapine, NFV – Nelfinavir, LPVr – Ritonavir-boosted Lopinavir, ABC – Abacavir, PI/r – Ritonavir-boosted Protease Inhibitor, TDF – Tenofovir Disoproxil Fumarate, FTC – Emtricitabine

Among age-and sex-matched HEU and HUU children, the linear regression's slope of LTL *vs.* age was similar in both groups (p=0.49) (Figure 2.6A). Comparison of LTL ranks (Mann Whitney U test) between the groups also showed no difference. In agreement with differences seen at birth, females had longer LTL than males, especially during the first 18 months of life. However, males maintained their LTL over time while females showed significant LTL attrition ($p<0.01$, Figure 2.6B-E).

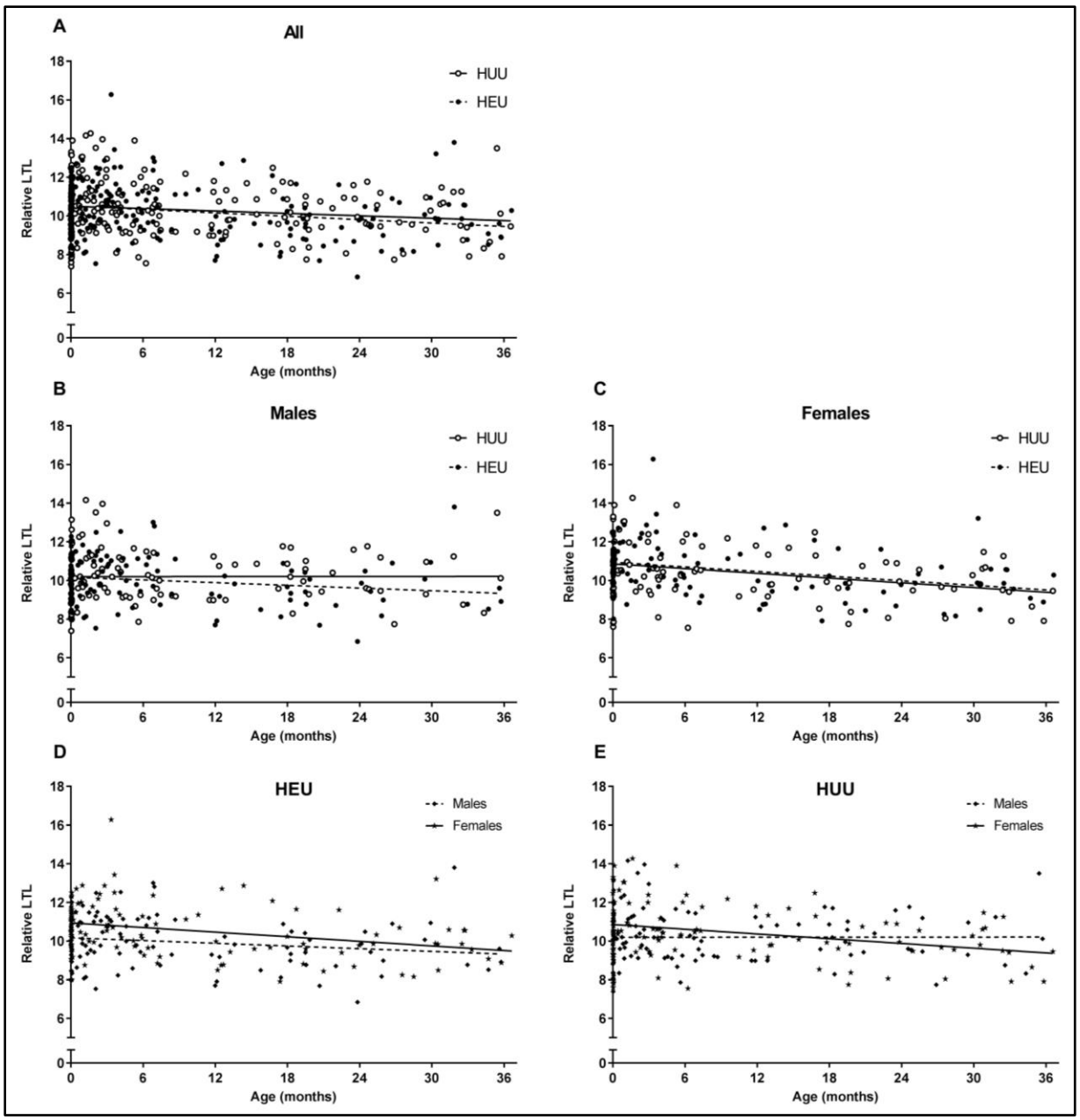

Figure 2.6. Relationship between LTL and age during the first three years of life among age- and sex-matched children. A – all HEU vs. all HUU. **B** – HEU males vs. HUU males. **C** – HEU females vs. HUU females. **D** – HEU males vs. HEU females. **E** – HUU males vs. HUU females.

2.3.4 Longitudinal LTL dynamics among HEU children

Longitudinal changes in LTL were assessed in the 214 HEU children for whom ≥ 2 blood specimens were collected between birth and 3 years. A significant non-linear relationship between LTL and age was observed, showing a rapid decline in LTL during the first 40 weeks (Figure 2.7). This was followed by a leveling out, perhaps even an upward trend, but the confidence intervals around the line are consistent with a flat relationship.

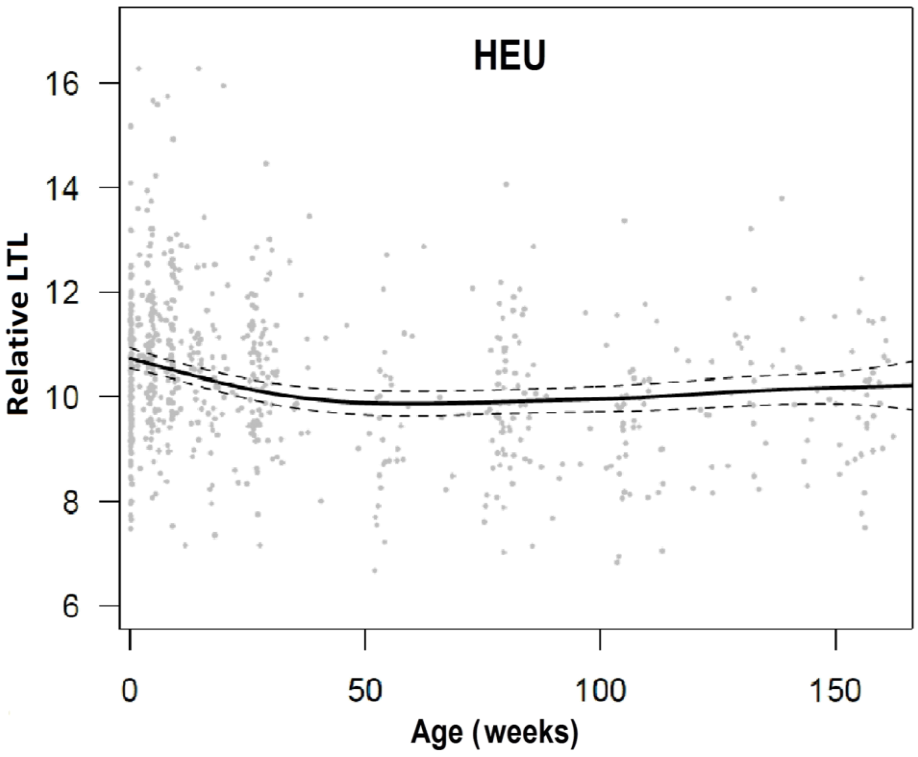

Figure 2.7. Non-linear regression model (generalized mixed effects additive model) of LTL and age (in weeks) for HEU infants. The solid and dashed lines indicate the regression line and the 95% CI of the estimated line, respectively. For clarity, one outlier (LTL value of 19.8) is omitted from the figure, but not from the analysis.

For the HUU infants for whom we had one specimen per participant, a linear regression model shows an inverse association between LTL and age (p=0.004, Figure 2.8).

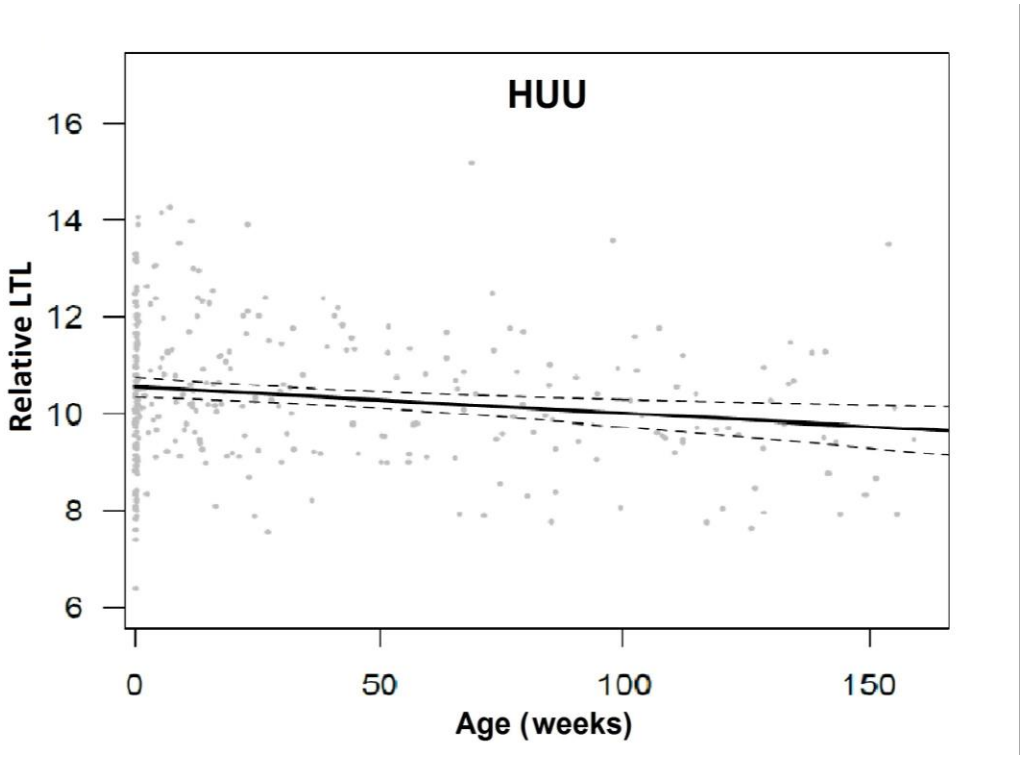

Figure 2.8. Linear regression model of LTL and age (in weeks) for HUU children. The solid and dashed lines indicate the regression line and the 95% CI of the estimated line, respectively.

2.3.5 HEU LTL during prophylaxis

Finally, to evaluate the possible effect of infant AZT prophylaxis on LTL, the change in HEU LTL between birth and the closest subsequent visit during prophylaxis was assessed using paired HEU WB specimens (Figure 2.9). There was no difference in LTL between birth and the following visit at a median [range] age of 31 [18-47] days (n=58, p=0.09).

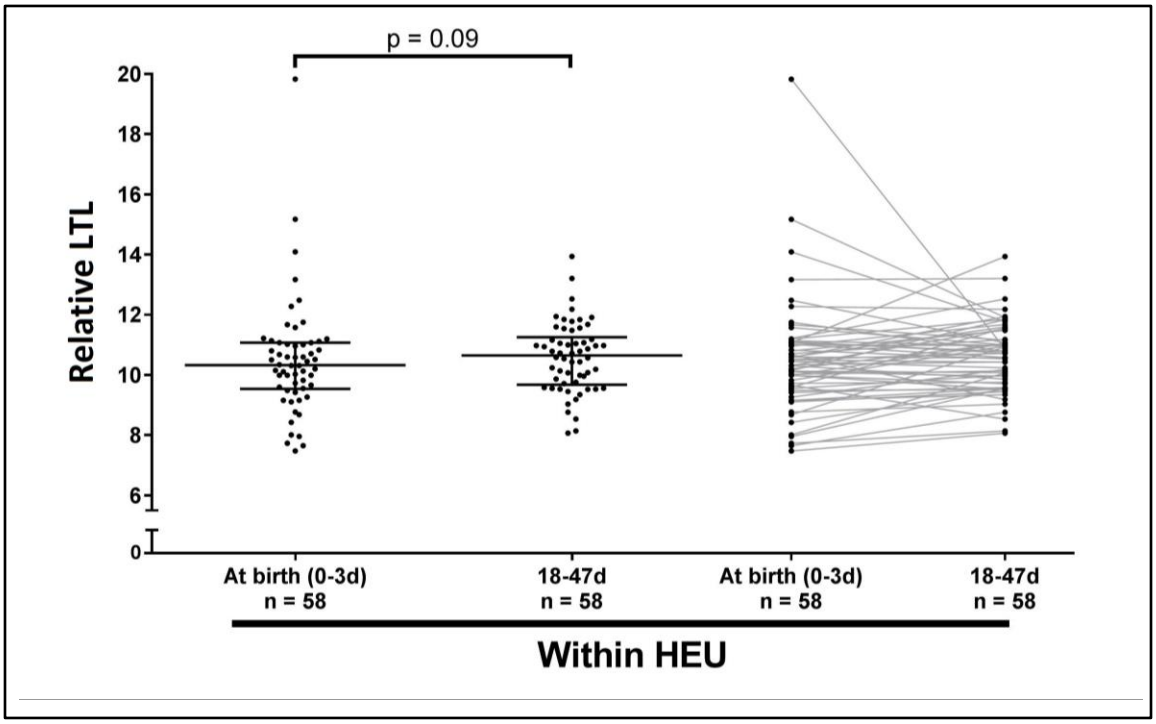

Figure 2.9. Comparisons of LTL at birth and the subsequent visit (18-47d) among HEU children (paired Wilcoxon signed-rank test)**.**

2.4 Discussion

In this cohort, we found no evidence that LTL are shorter in HEU compared to HUU, whether at birth, or over the first three years of life. These results are in agreement with smaller previous studies [78,79,244,245]. The HEU children's *in utero* cART exposure varied with respect to duration and type, in accordance with the treatment guidelines of the time [258,259]. HEU children exposed to maternal AZT+3TC+NFV or NVP had longer LTL at birth compared to HUU children. This may be related to the selective elimination of cell subsets with shorter telomeres and/or proliferation of cells with longer telomeres; our study was not designed to address this. Furthermore, HEU LTL did not change significantly during the AZT prophylaxis period. Taken together these results are reassuring and suggest that HIV/cART exposure appears to have little/no effect on infant telomere length early in life.

In contrast, maternal smoking during pregnancy does exert an effect on infant LTL at birth, albeit being in opposite directions in the two groups studied. Among HEU infants, being exposed to any maternal smoking *in utero* was associated with shorter LTL. This may be related to increased oxidative stress, in addition to stresses related to HIV/cART exposure. Although a similar effect was expected in HUU infants, our data instead suggest that HUU exposed to maternal smoking have longer LTL at birth compared to their unexposed peers. While this counterintuitive observation may be related to differences in smoking intensity, our exploration of this among a subset of women does not support this explanation. However, this is consistent with another study [260] that reported longer CD4+ T cell TL in infants born to smoking mothers. The mechanism behind this effect is unclear, and a larger prospective study with more details about smoking exposure and other confounders would be required to confirm this

observation. Early breastfeeding has been associated with longer LTL in children [261], and reduced breastfeeding rates and duration were reported among mothers who smoked [262]. We lacked breastfeeding information for our controls (~90% of HIV-uninfected women breastfeed in Canada), and none of the HIV+ mothers breastfed. Neither breastfeeding, nor the effects of smoking on breastfeeding are likely confounders in our study, given that the effects observed are in the opposite direction of those expected.

Within both HEU and HUU groups, female infants had longer LTL at birth compared to males, but also experienced faster attrition which attenuated any difference before three years of age. Our finding of longer TL among female infants is consistent with other studies that reported sex differences in LTL at birth and early childhood [263,264]. While it is well documented that women have longer telomeres than men [265–267], an effect attributed to the ability of estrogen to stimulate telomerase [268] and act as an antioxidant against ROS that damage telomeres [269], it is unclear how this is conferred as early as birth. Nevertheless, LTL early in life has been suggested to predict health outcomes later in life [263] and women do, on average, live longer than men.

Finally, we were uniquely positioned to investigate longitudinal changes in HEU LTL early in life. We observed that telomere attrition was rapid from birth to one year of age but slowed thereafter, mirroring those in a baboon study reporting a similar rapid decline in granulocyte and lymphocyte TL in the first year of life followed by a stabilization of TL [270]. This may reflect a period of significant growth, as well as differentiation and maturation of cells forming the innate and adaptive immune systems.

2.5 Strengths and Limitations

This is the first study to investigate longitudinal changes in children's LTL early in life. It is also the first detailed investigation of the potential effects of *in utero* exposure to maternal cART and smoking on infant LTL. However, our study has some limitations. Firstly, we lacked longitudinal samples from HUU infants that would have enabled detailed comparisons of LTL attrition dynamics between the HEU and HUU groups. Our cohort also had imbalanced ethnicity, with nearly half of the HEU but <1% of HUU children being Black/African Canadian. It is well known that Black individuals have longer telomeres than Whites [271–275]. Although our sensitivity analysis restricted to White children showed similar results, ethnicity could still be a confounding factor. Another limitation is the heterogeneity in smoking data collection across the three cohorts, which we addressed by defining smoking categorically, as yes/no ever during pregnancy. We acknowledge that this does not account for the smoking frequency or intensity, nor the duration, as some women may have quit during pregnancy. However, based on the subset of women for whom more extensive smoking information was available, the number of cigarettes per day reported was similar for the two groups. Furthermore, our qualitative data indicate that few women quit smoking and that the majority of women who smoked at any time in pregnancy continued smoking throughout their pregnancy. It is also possible that the observed association between smoking and LTL could be a proxy for the effect of an unmeasured confounder such as socioeconomic status or parental anxiety. Cytomegalovirus (CMV) infection is known to be associated with shorter telomeres [276], and we did not have access to information on CMV infection for our study participants. Paternal age is known to influence progeny TL, and this information was unavailable. Finally, given the small amount of blood

obtained, only LTL was measured, and our results cannot be generalized to TL in specific cell subsets as these may evolve substantially over the first years of life.

2.6 Conclusions

In conclusion, we found that LTL was similar between HEU and HUU children at birth, and over the first three years of life. Further, HIV/cART exposure *in utero* does not appear to alter telomere dynamics during early life, a reassuring finding. Instead, maternal smoking during pregnancy appears to exert a greater effect on infant LTL at birth, emphasizing the need for smoking cessation strategies, especially during pregnancy.

Chapter 3: THE EFFECTS OF *IN UTERO* EXPOSURE TO MATERNAL SMOKING ON INFANT TELOMERE LENGTH

3.1 Introduction

In North America, although smoking during pregnancy has been declining, an estimated 20-30% of women continue to engage in active smoking during pregnancy [277,278]. This can have serious health consequences for both mothers and developing fetuses. In addition to negatively affecting fecundity [279], smoking increases the prevalence of adverse pregnancy and infant outcomes such as spontaneous abortions [279], ectopic pregnancies [280], stillbirths [281], preterm birth and lower birth weight [282], and neonates who are small for gestational age [283]. The mechanism behind these adverse effects are unclear and likely multifactorial, but may be in part related to increased oxidative stress and placental dysfunction. In turn, newborns exposed to cigarette smoke *in utero* are at increased risk of developing age-related chronic diseases such as cardiovascular [284–286] and respiratory diseases [287,288], diabetes [289] , and certain cancers [290,291]. Such manifestations may also be a result of smoking-induced stress and oxidative damage to DNA.

There is a growing body of evidence linking smoking with shorter LTL in adults [60,292–296]. However, little is known about the effects of *in utero* exposure to maternal smoking on TL of infants. Almanzar *et al.* [260] recently reported longer CD4+ T cell TL in newborns of smoking mothers (n=58) compared to those born to non-smoking mothers (n=111). In contrast, another recent study by Salihu *et al.* [297] described a significant association

between smoking during pregnancy and infant cord blood TL, with shortest TL among active smokers (n=30), followed by passive smokers (n=31), and longest TL among non-smokers (n=25). As discussed in chapter two, our group recently reported longer infant LTL at birth among HUU children born to mothers who smoked during pregnancy [298]. Given this, I sought to investigate the association between maternal smoking during pregnancy and TL in various infant and maternal tissues.

3.2 Methods

3.2.1 Study design and study population

Study samples were collected as part of three Canadian cohort studies that included HIV-infected and HIV-uninfected (controls) pregnant women, as well as their infants: a pediatric cohort (births between 2004 and 2006) [255], a pregnancy cohort (births between 2005 and 2009) [245], and the Children and Women: AntiRetrovirals and Markers of Aging (CARMA) cohort (births between 2009 and 2011) (Figure 3.1) [79]. Control infants from these studies were included here if a birth blood specimen (0-3 days), as well as demographic, clinical and behavioral (maternal) characteristics were available (n=88). Available specimens from HIV-uninfected mothers in the latter two cohorts were also studied (n=52). This study constitutes secondary use of data and was approved by the University of British Columbia Clinical Research Ethics Board and the Children's and Women's Health Centre of BC Research Review Committee (H03-70356, H04-70540, H08-02018 and H09-02867) and all adult participants provided informed consent.

The following tissues were collected from the infants: umbilical cord blood (n=37) and cord tissue (n=18) at birth, heel-prick whole blood (into a BD pediatric EDTA tube (n=88)), and mouth swab epithelial cells (n=18) collected by a sterile swab rub of the inner cheek, taken at 0-3 days of life. Similarly, maternal peripheral venous blood (n=50) was collected between 31 and 40 weeks of gestation by arm venipuncture, at the same time as the maternal mouth swab (n=27). Placental tissue from both fetal and maternal sides of the organ (n=54 each) were collected shortly after delivery and flash frozen into liquid nitrogen. All tissues were stored at -80°C until DNA extraction.

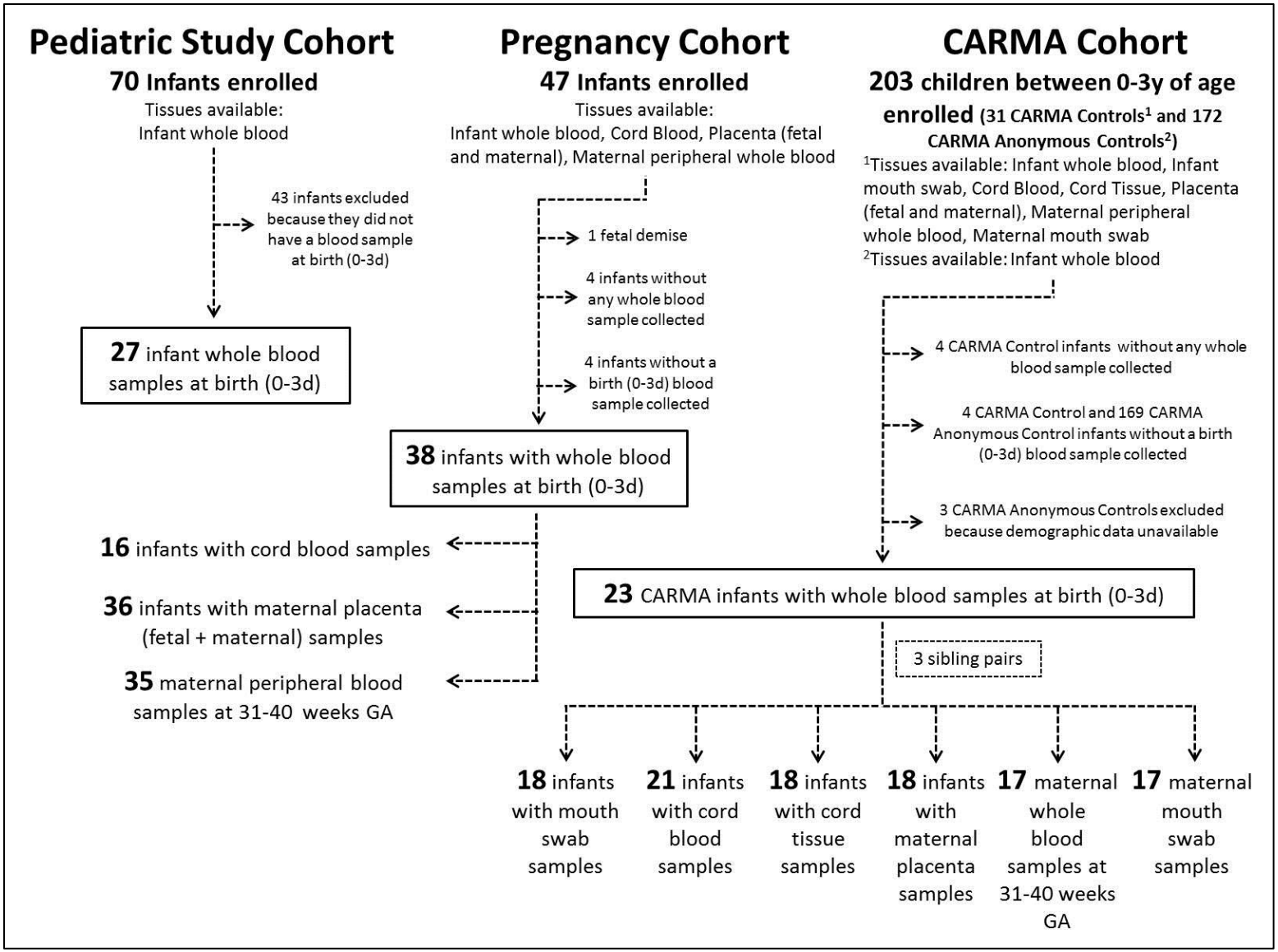

Figure 3.1. Schematic of study participants.

3.2.2 DNA extraction and relative TL measurements

Total genomic DNA was extracted from 0.1ml of infant, maternal, or cord blood using the QIAamp® DNA mini kit (Qiagen) on a QIAcube according to the "Blood and Body fluids" protocol with the following modifications: blood samples were diluted 1:1 with 0.1ml phosphate-buffered saline (PBS) prior to extraction and DNA was eluted into a final volume of 100µl AE buffer. DNA from mouth swab samples was extracted using the same kit (Qiagen) according to the "Buccal Swab Spin" protocol. For cord tissue DNA extraction, 20-25 mg of the tissue was cut into small pieces and homogenized in 360µl of lysis buffer (Buffer ATL, Qiagen). The QIAamp® DNA mini kit (Qiagen) "Tissue protocol" was followed for manual extraction of DNA with the following modifications: the volumes of proteinase K, buffer AL and ethanol were doubled and a proteinase K overnight incubation was carried out to achieve complete tissue lysis. DNA extraction from 25mg of the placental tissue was done using the AllPrep DNA/RNA kit (Qiagen) according to the "Simultaneous purification of Genomic DNA and Total RNA from Animal Tissues" protocol. Relative TL was then quantified via monochrome multiplex quantitative polymerase chain reaction (qPCR) as previously described [257].

3.2.3 Statistical analyses

The χ^2 test, Student's *t* test, and Mann-Whitney test were used to compare clinical and demographic characteristics of the study participants. Univariate linear regression modelling was used to investigate associations between infant TL and the following explanatory variables: infant sex, GA at birth, preterm birth (GA <37 weeks), birth weight, SGA, maternal age at birth, maternal ethnicity, and maternal smoking ever during pregnancy. For the maternal tissues, explanatory variables included: maternal age, ethnicity, history of hepatitis C virus (HCV)

infection, income (<CAD $15,000/year *vs.* >CAD $15,000/year), GA at visit, preterm delivery, maternal smoking (cigarettes and/or marijuana), alcohol use, and other illicit substance use since last visit. Due to heterogeneity in data collection between cohorts, smoking herein refers to self-reported maternal smoking ever during pregnancy, recorded as a categorical yes *vs.* no, irrespective of the intensity, frequency, and duration of cigarette smoking. Maternal plasma was not available from the majority of study participants. However, the accuracy of self-reported smoking data was internally validated by evaluating concordance between self-reported smoking and plasma cotinine levels in an independent set of women enrolled in these same cohorts (see section 3.2.4). Variables important univariately ($p<0.15$) were considered when developing multivariable models. Collinearity between variables was determined by examining contingency tables. TL relationships between tissues were examined using Pearson's and Spearman's correlations.

3.2.4 Validation of self-reported smoking status (Cotinine analysis)

To validate self-reported smoking status by pregnant women, plasma cotinine levels were measured in samples collected during the third trimester of pregnancy (31-37 weeks of gestation) from a total of 47 HIV+ and HIV- women enrolled in the CARMA cohort. Substance use information including smoking was collected by self-report on the same day as blood collection. Cotinine was measured by solid phase competitive enzyme-linked immunosorbent assay (ELISA, Calbiotech, US) according to the manufacturer's instructions. All plasma samples were treated with 1% Triton X-100 at room temperature for 4 hours to deactivate viruses prior to assaying. The proportions of cotinine-negative among self-reported non-users and of cotinine-positive among self-reported users were used to express concordance.

Among the 47 women included in this analysis, 26% reported smoking daily, 4% weekly, and 55% reported being non-smokers. There was no frequency data available for the remaining 15% who did report smoking. Defining smoking by a plasma cotinine cut-off of 5 ng/ml, we observed a 90% concordance between self-reported smoking (any smoking since last pregnancy visit) and plasma cotinine. For the self-reported non-smokers, concordance was 88%. Two pregnant women classified as smokers according to their self-report data (yes vs. no) showed plasma cotinine level <5 ng/ml but a closer inspection revealed that they reported smoking on average fewer than 2 cigarettes per week during their pregnancy. Given the half-life of cotinine, the timing of maternal sampling could influence its detection. If these two women are re-classified as non-smokers, the concordance between cotinine and the self-reported smoking or non-smoking rises to 100% and 89%, respectively.

3.3 Results

3.3.1 Characteristics of study participants

All 88 infants included in this study were singleton. If a mother had more than one pregnancy within one or more of the cohorts, all her infants were included. There were three pairs of siblings. For maternal analyses, data from the first pregnancy was considered. Forty three percent (43%) of pregnant women smoked during pregnancy. As seen in Table 3.1, smokers (S) were younger, and more likely to be of Aboriginal/Indigenous ethnicity than the non-smoking (NS) comparators. The infants of women who smoked during pregnancy were more likely to be born preterm (<37 weeks gestation), with a lower birth weight, but were not more likely to be SGA. There was no difference in infant sex between groups. Among the 52 mothers who had biological specimen available, those who self-reported smoking in the period

preceding their study visit (which took place between 31-40 weeks of gestation) were more likely to be young, of Aboriginal/Indigenous ethnicity, to have an income <CAD $15,000/year, to have used illicit drugs during pregnancy, and to have a history of HCV compared to non-smokers (Table 3.2).

Table 3.1. Demographic and clinical characteristics of infants born to smoking and non-smoking mothers.

	Smokers (S) (n = 38)	Non-smokers (NS) (n = 50)	p value
Infant Characteristics			
Male sex	17 (45)	30 (60)	0.20
GA, weeks	38.7 (28.9 - 41.9)	39.9 (36.6- 42.1)	**0.003**
< 37 weeks	8 (21)	1 (2)	
< 34 weeks	1 (3)	0 (0)	
Birth weight, kg	3.3 (1.4 - 5.2)	3.6 (2.6 - 4.9)	**0.007**
SGA	6 (16)	6 (12)	0.76
Maternal Characteristics			
Maternal age at birth, years	30.1 (21.3 - 41.3)	34.2 (22.8 - 43.0)	**< 0.001**
Maternal ethnicity			**0.002**
Aboriginal/Indigenous	7 (18)	0 (0)	
Black/African Canadian	1 (3)	1 (2)	
White	25 (66)	30 (60)	
Asian	4 (10)	11 (22)	
Other/Unknown	1/0 (3)	1/7 (16)	

Data are presented as n (%) or median (range). Abbreviations: GA – Gestational Age, SGA – Small for GA

Table 3.2. Demographic and clinical characteristics of smoking and non-smoking mothers with a study visit before delivery, between 31-40 weeks of gestation.

	Smoking at visit (n = 19)	Non-smoking at visit (n = 33)	p value
Maternal age, years	27.4 (22.2 - 36.2)	34.2 (21.3 - 43.0)	**< 0.001**
Maternal ethnicity			0.21
Aboriginal/Indigenous	5 (26)	2 (6)	
Black/African Canadian	0 (0)	2 (6)	
White	11 (58)	23 (70)	
Asian	3 (16)	6 (18)	
Other/Unknown	0 (0)	0 (0)	
GA at visit, weeks	34.1 (31.9 - 39.7)	34.7 (32.1 - 37.9)	0.12
Preterm birth (< 37 weeks GA)	3 (16)	3 (9)	0.66
Income < $15,000/year	15 (79)	5 (15)	**< 0.001**
History of HCV infection	6 (32)	0 (0)	**0.001**
Alcohol at visit	1 (5)	6 (18)	0.24
Illicit* drug use at visit	8 (42)	0 (0)	**< 0.001**

Data are presented as n (%) or median (range). Abbreviations: GA – Gestational Age, HCV – Hepatitis C Virus.
*Illicit drug includes heroin, cocaine, opioids, amphetamines, and/or benzodiazepene

3.3.2 Univariate and Multivariable Regression Analyses

3.3.2.1 Infant LTL at birth

A total of 86 infant LTL measures were considered for the analyses as two infant blood extracts contained insufficient DNA to meet assay QC. Table 3.3 describes the results of the univariate and multivariable analyses of LTL at birth. Female sex (p=0.03) and maternal smoking in pregnancy (p=0.002) were significantly associated with longer infant birth LTL. Preterm birth showed some association with longer infant LTL, although it did not reach significance (p=0.06). Unadjusted comparisons of TL between the S and NS groups are shown in Figure 3.2.

Table 3.3. Univariate and Multivariable regression analyses of the association between possible predictors and infant leukocyte telomere length (LTL).

Explanatory variables	Infant LTL n = 86 (37 S, 49 NS)					
	Univariate			Multivariable		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Infant Sex, Male (ref. Female)	-0.23	[-0.44 to -0.02]	**0.03**	-0.19	[-0.39 to -0.02]	0.076
GA, weeks	-0.11	[-0.33 to 0.11]	0.31	---	---	---
Preterm Birth (< 37w GA), Yes (ref. No)	0.21	[-0.004 to 0.42]	0.06	---	---	---
Birth Weight, kg	-0.04	[-0.25 to 0.18]	0.74	---	---	---
SGA, Yes (ref. No)	-0.03	[- 0.25 to 0.19]	0.79	---	---	---
Maternal age	-0.12	[-0.33 to 0.10]	0.29	0.11[b]	[-0.21 to 0.23]	0.93
Maternal ethnicity (ref. White)				---	---	---
Aboriginal/Indigenous	0.10	[-0.13 to 0.33]	0.38	---	---	---
Black/African Canadian	-0.06	[-0.29 to 0.17]	0.63	---	---	---
Asian and Others	-0.002	[-0.23 to 0.23]	0.99	---	---	---
Maternal smoking ever in pregnancy, Yes (ref. No)[a]	0.33	[0.12 to 0.53]	**0.002**	0.30	[0.09 to 0.51]	**0.005**

Abbreviations: GA – Gestational Age, S – Smokers, NS – Non-smokers, SGA – Small for GA

[a]Preterm birth and maternal smoking ever in pregnancy were collinear variables and hence the final multivariable model included only maternal smoking and infant sex

[b]When maternal age at birth was forced in the model, maternal smoking ever in pregnancy remained significantly associated with longer infant LTL (β = 0.30, 95%CI = [0.08 to 0.53], **p = 0.009**), while neither female sex (β = 0.18, 95%CI = [-0.02 to 0.39], p = 0.08) nor maternal age at birth (β = 0.11, 95%CI = [-0.21 to 0.23], p = 0.93) were significantly associated.

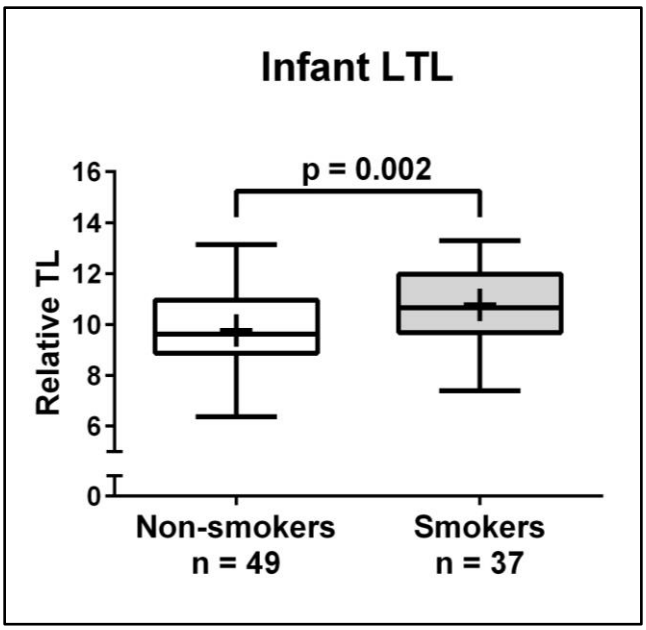

Figure 3.2. Unadjusted comparisons of LTL between the S and NS groups.

Collinearity was observed between maternal smoking during pregnancy and preterm birth (Table 3.4) and hence these were investigated in turn while developing multivariable models.

Table 3.4. Contingency tables describing the collinearity between maternal smoking and preterm birth among participants with LTL data.

	Maternal smoking ever in pregnancy – Yes	Maternal smoking ever in pregnancy – No	Total
Preterm birth - Yes	8	1	9
Preterm birth - No	30	49	79
Total	38	50	88
p = 0.005			

In the final model (Figure 3.3A, Table 3.3) that included only infant sex and maternal smoking ever in pregnancy, smoking remained significantly associated with longer infant birth LTL (p=0.005), while female sex did not reach significance (p=0.076). Smoking mothers were significantly younger than non-smoking mothers (p<0.001), however, the results did not change when maternal age at birth was forced into the multivariable model (Table 3.3 footnote).

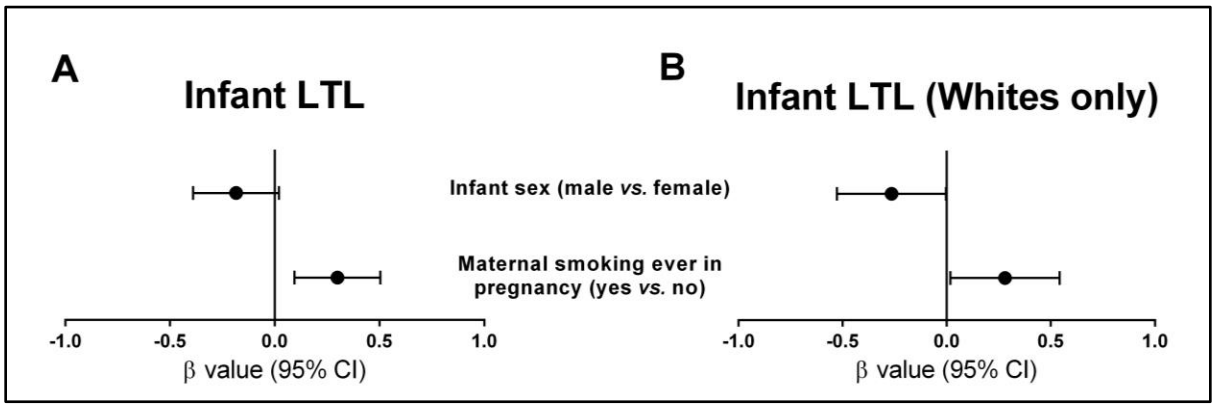

Figure 3.3. Multivariable regression analyses of the association between possible predictors and infant LTL at birth among (A) all infants, and (B) infants born to White mothers only. Effect size (β values) and 95% confidence intervals are shown for possible predictors.

An imbalance in ethnicity was present between the groups whereby all Aboriginal/Indigenous participants were smokers, while there were more Asians in the non-smoking group (Table 3.5).

Table 3.5. Contingency tables describing collinearity between maternal smoking and ethnicity among participants with LTL data

	Maternal smoking ever in pregnancy – Yes	Maternal smoking ever in pregnancy – No	Total
Aboriginal/Indigenous	7	0	7
Black/African Canadian	1	1	2
Asian and others	5	12	17
White	25	30	55
Total	38	43	81
Frequency missing = 7			
p = 0.007			

To address this possible confounder, a sensitivity analysis was performed that included only infants born to White mothers (total n=54, 24 S and 30 NS). As before, univariate regression analyses (Table 3.6) revealed that female sex (p=0.01) and maternal smoking during pregnancy (p=0.01) were significantly associated with longer infant birth LTL. These associations remained significant in the multivariable model (Figure 3.3B, Table 3.6). However, when maternal age was forced into this model, the effect of smoking during pregnancy was weakened (p=0.09), and only female sex (p=0.04) remained significantly associated with longer infant LTL at birth (Table 3.6 footnote).

Table 3.6. Univariate and Multivariable regression analyses of the association between possible predictors and infant LTL among infants born to White mothers only (sensitivity analysis).

Explanatory variables	Infant LTL n = 54 (24 S, 30 NS)					
	Univariate			Multivariable		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Infant Sex, Male (ref. Female)	-0.34	[-0.60 to -0.07]	**0.01**	-0.27	[-0.53 to -0.003]	**0.047**
GA, weeks	-0.09	[-0.37 to 0.19]	0.52	---	---	---
Preterm Birth (< 37w GA), Yes (ref. No)	0.22	[-0.05 to 0.50]	0.10	---	---	---
Birth Weight, kg	-0.004	[-0.28 to 0.27]	0.98	---	---	---
SGA, Yes (ref. No)	-0.15	[- 0.43 to 0.12]	0.27	---	---	---
Maternal age	-0.16	[-0.44 to 0.11]	0.2	-0.10[a]	[-0.37 to 0.17]	0.47
Maternal smoking ever in pregnancy, Yes (ref. No)	0.35	[0.09 to 0.61]	**0.01**	0.28	[0.02 to 0.54]	**0.037**

Abbreviations: GA – Gestational Age, S – Smokers, NS – Non-smokers, SGA – Small for GA

[a]When maternal age at birth was forced in the model, the effect of maternal smoking ever in pregnancy was weakened (β = 0.24, 95%CI = [-0.04 to 0.53], p = 0.089), and only female sex (β = 0.28, 95%CI = [0.01 to 0.55], **p = 0.04**) remained significantly associated with longer infant LTL at birth.

To determine whether preterm birth may be confounding the observed association, another sensitivity analysis that included only infants born at term (n=77, 29S and 48 NS) was performed. Variables univariately associated were similar. In the multivariable model, female sex (p=0.035) and smoking during pregnancy (p=0.02) remained independently associated with longer infant LTL at birth (Table 3.7).

Table 3.7. Univariate and Multivariable regression analyses of the association between possible predictors and infant LTL among infants born at term (sensitivity analysis).

Explanatory variables	Infant LTL n = 77 (29 S, 48 NS)					
	Univariate			Multivariable		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Infant Sex, Male (ref. Female)	-0.27	[-0.50 to -0.05]	**0.02**	-0.23	[-0.45 to -0.02]	**0.035**
GA, weeks	-0.007	[-0.24 to 0.22]	0.95	---	---	---
Birth Weight, kg	0.03	[-0.20 to 0.26]	0.77	---	---	---
SGA, Yes (ref. No)	<0.001	[- 0.23 to 0.23]	0.99	---	---	---
Maternal age	-0.12	[-0.35 to 0.11]	0.29	-0.01[a]	[-0.25 to 0.22]	0.92
Maternal ethnicity (ref. White)				---	---	---
Aboriginal/First Nation	0.08	[-0.16 to 0.33]	0.50	---	---	---
Black/African Canadian	-0.05	[-0.29 to 0.20]	0.70	---	---	---
Asian and Others	0.01	[-0.24 to 0.26]	0.92	---	---	---
Maternal smoking ever in pregnancy, Yes (ref. No)	0.30	[0.08 to 0.52]	**0.008**	0.26	[0.05 to 0.48]	**0.02**

Abbreviations: GA – Gestational Age, S – Smokers, NS – Non-smokers, SGA – Small for GA

[a]When maternal age at birth was forced in the model, both maternal smoking ever in pregnancy (β = 0.26, 95%CI = [0.02 to 0.50], **p = 0.037**) and female sex (β = 0.24, 95%CI = [0.02 to -0.45], **p = 0.036**) remained significantly associated with longer infant LTL.

3.3.2.2 Other Infant tissues - Mouth swab, Cord blood and Cord tissue TL

The singular observation of longer LTL in infants exposed to smoking *in utero* triggered the exploration of TL in other tissues available from a subset of these infants. A total of 18 mouth swab (8 S and 10 NS), 37 cord blood (19 S and 18 NS) and 18 cord tissue (9 S and 9 NS) DNA samples were additionally assayed for relative TL. Univariate analyses (Table 3.8) of the same explanatory variables suggested that maternal smoking was also significantly associated with longer TL in mouth swab (p=0.003) and cord blood (p=0.047). In cord tissue, only female sex (p=0.017) showed an association with longer TL. The unadjusted comparisons between S and NS are shown in Figure 3.4, and the multivariable models for cord blood and cord tissue TL are shown in Figure 3.5.

Table 3.8. Univariate regression analyses of the association between possible predictors and TL in other infant tissues.

Explanatory variables	Infant Mouth Swab Log TL			Cord Blood TL			Cord Tissue TL		
	n = 18 (8 S, 10 NS)			n = 37 (19 S, 18 NS)			n = 18 (9 S, 9 NS)		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Infant Sex, Male (ref. Female)	-0.21	[-0.72 to 0.31]	0.41	-0.31	[-0.63 to 0.02]	0.07	-0.55	[-0.99 to -0.11]	**0.017**
GA, weeks	0.09	[-0.43 to 0.62]	0.71	-0.12	[-0.53 to 0.15]	0.26	-0.24	[-0.76 to 0.27]	0.34
Preterm Birth (< 37w GA), Yes (ref. No)	-0.12	[-0.64 to 0.41]	0.65	0.14	[-0.21 to 0.48]	0.43	0.10	[-0.43 to 0.63]	0.70
Birth Weight, kg	0.11	[-0.41 to 0.64]	0.65	0.11	[-0.41 to 0.64]	0.65	-0.07	[-0.60 to 0.46]	0.77
SGA, Yes (ref. No)	---	---	---	---	---	---	---	---	---
Maternal age, years	-0.17	[-0.69 to 0.36]	0.51	-0.17	[-0.69 to 0.36]	0.51	0.22	[-0.30 to 0.74]	0.38
Maternal ethnicity, Non-white (ref. White)	0.57	[0.14 to 1.01]	**0.013**	-0.05	[-0.39 to 0.29]	0.78	0.11	[-0.42 to 0.64]	0.66
Maternal smoking ever in pregnancy, Yes (ref. No)	0.66	[0.26 to 1.06]	**0.003**	0.33	[0.004 to 0.65]	**0.047**	0.34	[-0.16 to 0.84]	0.17

Abbreviations: GA – Gestational Age, HCV – Hepatitis C Virus, S – Smokers, NS – Non-smokers, SGA – Small for GA

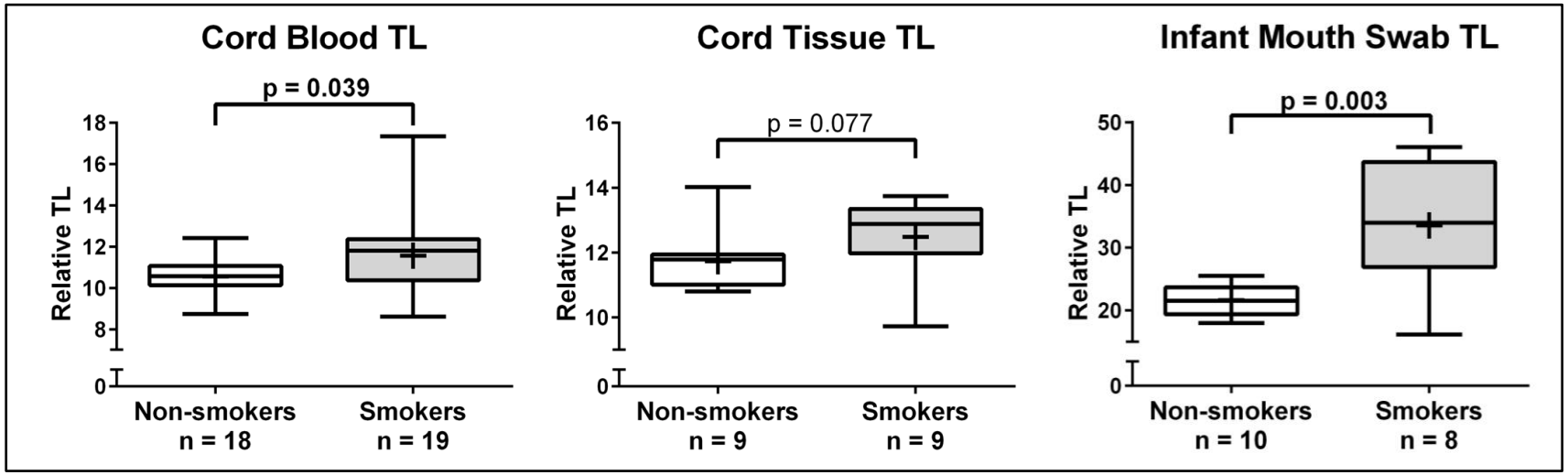

Figure 3.4. Unadjusted comparisons of cord blood, cord tissue, and mouth epithelial cell TL between the S and NS groups.

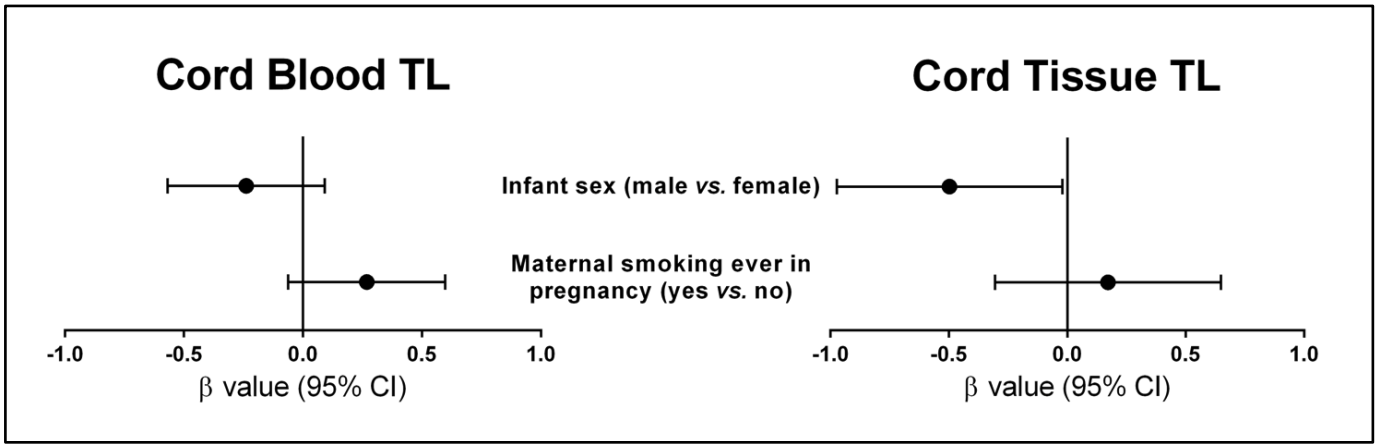

Figure 3.5. Multivariable regression analyses of the association between possible predictors and infant cord blood TL and cord tissue TL. Effect size (β values) and 95% confidence intervals are shown for possible predictors.

3.3.2.3 Placental TL

DNA was extracted from the maternal (P/M) and fetal (P/F) sides of 54 placentae (20 S and 34 NS) and assayed for TL. Given the placenta's feto-maternal origin, combinations of both infant and maternal variables were considered for their association with TL on either side of the organ (Table 3.9). Univariately, both preterm delivery (P/F: p=0.007, P/M: p=0.01) and smoking during pregnancy (P/F: p=0.04, P/M: p=0.01) were significantly associated with longer placental TL. Unadjusted comparisons of placental TL between S and NS groups are shown in Figure 3.6.

Table 3.9. Univariate regression analyses of the association between possible predictors and placental TL (fetal and maternal sides).

Explanatory variables	Placenta – Fetal Log TL			Placenta – Maternal Log TL		
	n = 54 (20 S, 34 NS)			n = 54 (20 S, 34 NS)		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Infant Sex, Male (ref. Female)	-0.05	[-0.33 to 0.23]	0.74	0.04	[-0.24 to 0.31]	0.80
GA, weeks	-0.14	[-0.41 to 0.14]	0.33	-0.17	[-0.45 to 0.10]	0.21
Preterm Birth (< 37w GA), Yes (ref. No)	0.37	[0.11 to 0.64]	**0.007**	0.34	[0.08 to 0.61]	**0.01**
Birth Weight, kg	-0.15	[-0.43 to 0.13]	0.28	-0.16	[-0.43 to 0.12]	0.26
SGA, Yes (ref. No)	0.24	[-0.03 to 0.51]	0.08	0.22	[-0.05 to 0.49]	0.11
Maternal age, years	-0.11	[-0.38 to 0.17]	0.45	-0.02	[-0.30 to 0.26]	0.90
Maternal ethnicity, Non-white (ref. White)	0.22	[-0.06 to 0.49]	0.12	0.23	[-0.04 to 0.50]	0.09
Maternal smoking ever in pregnancy, Yes (ref. No)	0.29	[0.02 to 0.55]	**0.04**	0.34	[0.08 to 0.60]	**0.01**
Maternal history of HCV, Yes (ref. No)	0.22	[-0.07 to0.50]	0.13	0.22	[-0.06 to 0.50]	0.13

Abbreviations: GA – Gestational Age, HCV – Hepatitis C Virus, S – Smokers, NS – Non-smokers, SGA – Small for GA

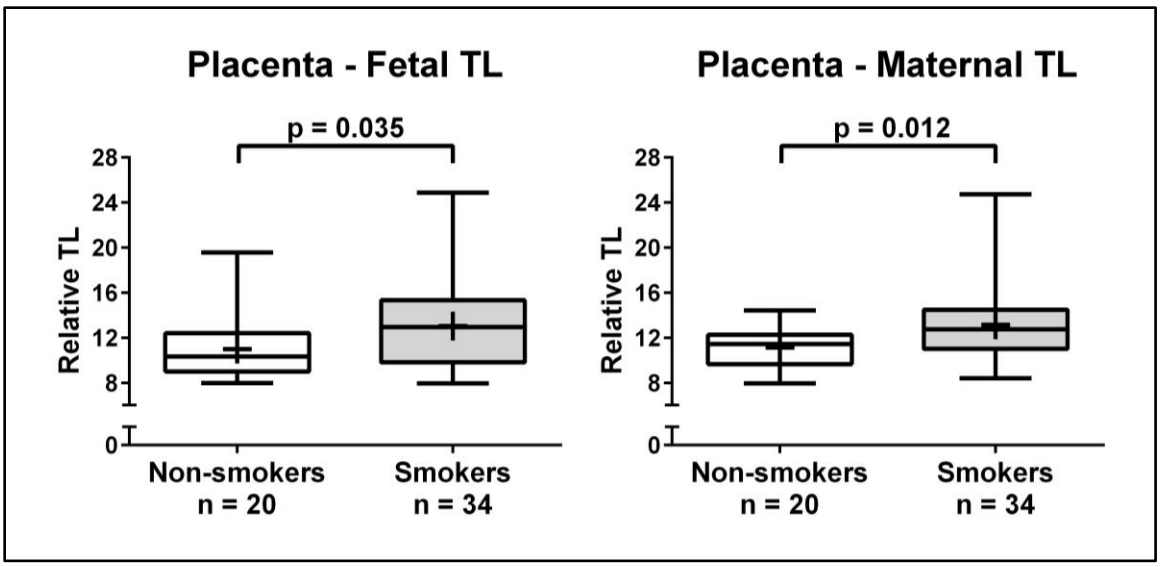

Figure 3.6. Unadjusted comparisons of placental TL between S and NS groups

As seen in Table 3.10, preterm birth and smoking during pregnancy were highly collinear. Therefore, multivariable models were developed for placental TL, with smoking and preterm birth considered in turn. In the final model (Table 3.11 and Figure 3.7) smoking during pregnancy lost its association and was removed, but preterm birth remained significantly associated with longer placental TL (P/F: p=0.003, P/M: p=0.004). Of note, the result did not change when smoking during pregnancy was added back into this last model (Table 3.11 footnote). However, this finding may be spurious given the absence of preterm deliveries among non-smoking mothers for whom placenta was collected.

Table 3.10. Contingency tables describing collinearity between preterm birth and smoking among participants with placenta TL data.

	Maternal smoking ever in pregnancy – Yes	Maternal smoking ever in pregnancy – No	Total
Preterm birth - Yes	7	0	7
Preterm birth - No	27	20	47
Total	34	20	54
p = 0.038			

Table 3.11. Multivariable regression analyses of the association between possible predictors and placental TL (fetal and maternal sides).

Explanatory variables	Placenta – Fetal Log TL			Placenta – Maternal Log TL		
	n = 54 (20 S, 34 NS)			n = 54 (20 S, 34 NS)		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Infant Sex, Male (ref. Female)	---	---	---	---	---	---
Preterm Birth (< 37w GA), Yes (ref. No)*	0.41	[0.14 to 0.67]	**0.003**	0.40	[0.13 to 0.66]	**0.004**
SGA, Yes (ref. No)	0.23	[-0.06 to 0.52]	0.11	0.20	[-0.10 to 0.49]	0.18
Maternal ethnicity, Non-white (ref. White)	0.09	[-0.20 to 0.38]	0.53	0.11	[-0.18 to 0.40]	0.45
Maternal smoking ever in pregnancy, Yes (ref. No)*	---	---	---	---	---	---
Maternal history of HCV, Yes (ref. No)	0.16	[-0.10 to 0.43]	0.18	0.22	[-0.09 to 0.45]	0.20

Abbreviations: GA – Gestational Age, S – Smokers, NS – Non-smokers, SGA – Small for GA

*For the Placenta analyses, preterm birth and maternal smoking ever in pregnancy were collinear variables. Maternal smoking was not independently associated with placenta TL in the multivariable models investigated. The final multivariable model shown in the table considered preterm birth, small for GA, maternal ethnicity and history of HCV for possible inclusion in the model based on univariate associations. The results did not change when smoking was added to this model

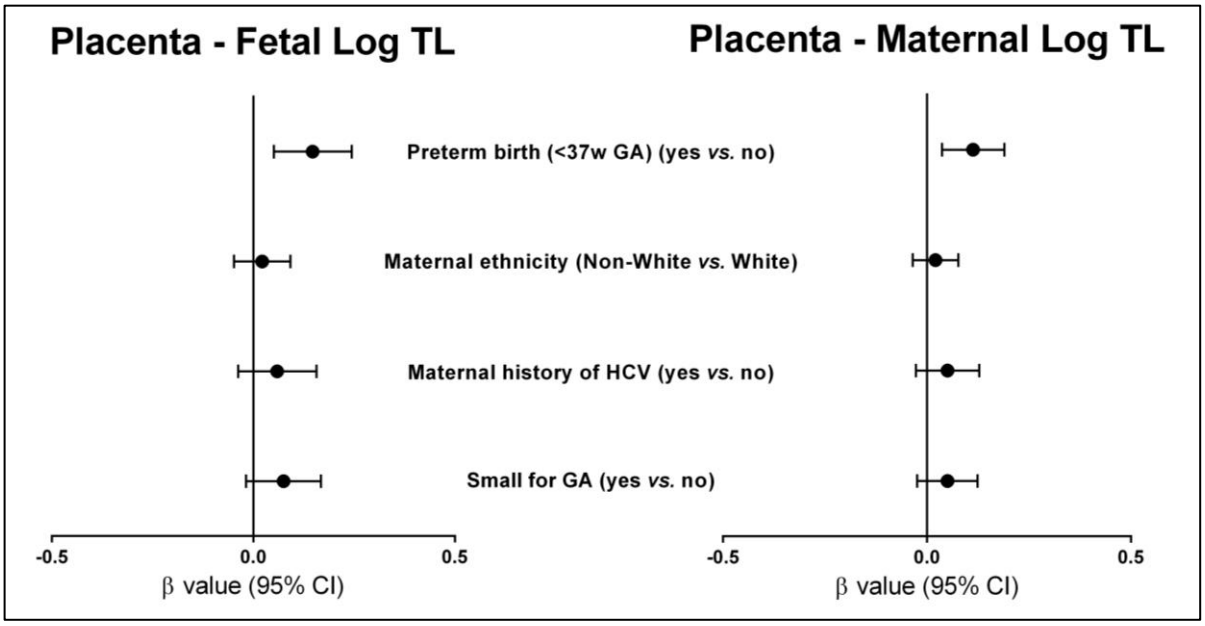

Figure 3.7. Multivariable regression analyses of the association between possible predictors and placental TL (fetal and maternal sides). Effect size (β values) and 95% confidence intervals are shown for possible predictors.

3.3.2.4 Maternal LTL and mouth swab TL between 31-40 weeks of gestation

Among the 52 mothers with a blood specimen, 17 had a mouth swab specimen (5 S and 12 NS) collected on the same day as blood. None of the possible explanatory variables investigated were univariately associated with maternal LTL or mouth swab TL (Table 3.12). The strongest association observed was between preterm delivery and longer maternal LTL (p=0.06). Power was insufficient to carry out multivariable modeling for maternal tissues. Unadjusted comparisons of maternal LTL and mouth swab TL between S and NS are shown in Figure 3.8. The graphs suggest a possible association between maternal smoking and shorter TL in mouth epithelial cells, but the small sample size limits this comparison.

Table 3.12. Univariate regression analyses of the association between possible predictors and (a) maternal LTL, (b) maternal mouth swab TL.

Explanatory variables	Maternal LTL			Maternal Mouth Swab TL		
	n = 52 (19 S, 33 NS)			n = 17 (5 S, 12 NS)		
	Effect size β	95% CI	p value	Effect size β	95% CI	p value
Maternal age, years	-0.07	[-0.36 to 0.21]	0.60	0.04	[-0.51 to 0.59]	0.87
Maternal ethnicity, Non-White (ref. White)	-0.13	[-0.41 to 0.15]	0.36	-0.13	[-0.67 to 0.42]	0.63
GA at visit, weeks	-0.07	[-0.36 to 0.21]	0.60	0.04	[-0.51 to 0.59]	0.88
Preterm Birth, Yes (ref. No)	0.27	[-0.01 to 0.54]	0.06	---	---	---
Income (< $15,000 vs. > $15,000)	0.05	[-0.24 to 0.33]	0.75	-0.38	[-0.89 to 0.13]	0.13
History of HCV, Yes (ref. No)	0.04	[-0.25 to 0.32]	0.81	---	---	---
Illicit drugs at visit	0.04	[-0.25 to 0.32]	0.79	---	---	---
Alcohol at visit	0.007	[-0.28 to 0.29]	0.96	---	---	---
Maternal smoking at visit, Yes (ref. No)	-0.04	[-0.32 to 0.25]	0.78	-0.43	[-0.92 to 0.07]	0.09

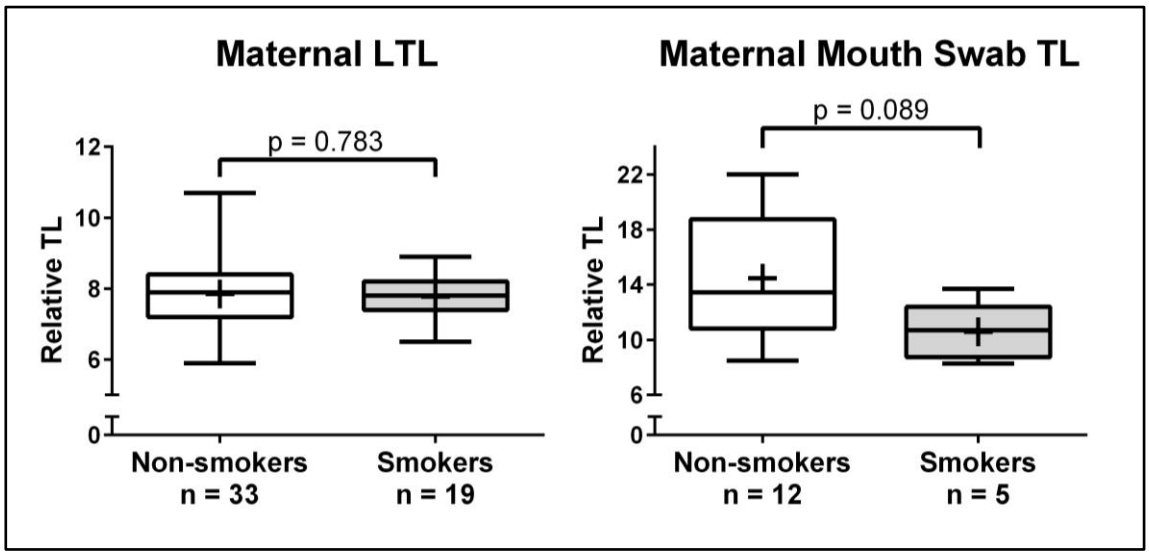

Figure 3.8. Unadjusted comparisons of maternal LTL and maternal mouth epithelial cell TL between S and NS groups.

3.3.2.5 Correlation between infant tissues

Consistent with others [299], we observed that the TL at birth was generally synchronized among the various infant tissues. The relationship(s) between TL in different infant and maternal tissues is summarized in Table 3.13. As several datasets were not normally distributed, Spearman's correlations were used in most cases. Significant correlations were observed between infant LTL at birth and TL in the following tissues: cord blood and cord tissue, placenta (both fetal and maternal) and maternal mouth swab. Infant LTL was not significantly correlated with infant mouth swab TL, or with maternal LTL between 31-40 weeks GA. For the subset of study participants for whom TL measurements were available in all 8 tissues (n=12, 6 S and 6 NS), Figure 3.9 illustrates the intra-individual TL patterns across tissues. It illustrates the strikingly opposite effect of smoking on mouth swab TL in mothers versus their infants.

Table 3.13. Correlations between TL in infant and maternal tissues.

	Infant LTL	Infant Mouth Swab TL	Cord Blood TL	Cord Tissue TL	Placenta - Fetal TL	Placenta - Maternal TL	Maternal LTL	Maternal Mouth Swab TL	
Infant LTL	---	0.10	0.52	0.51*	0.45	0.58	0.02*	-0.61*	**Correlation Coefficients (r/rho)**
Infant Mouth Swab TL	0.68	---	0.49	0.42	0.009	0.47	-0.24	-0.46	
Cord Blood TL	**0.002**	**0.05**	---	0.77	0.47	0.45	0.16	-0.58	
Cord Tissue TL	**0.03***	0.11	**<0.001**	---	0.20	0.27	-0.24*	-0.66*	
Placenta - Fetal TL	**0.001**	0.97	**0.006**	0.43	---	0.80	0.15	-0.15	
Placenta - Maternal TL	**< 0.001**	0.07	**0.010**	0.28	**< 0.001**	---	0.03	-0.27	
Maternal LTL	0.88*	0.42	0.37	0.41*	0.31	0.84	---	0.49*	
Maternal Mouth Swab TL	**0.009***	0.10	**0.02**	**0.011***	0.62	0.36	**0.045***	---	
	p values								

Correlations presented are mostly Spearman's since data was not normally distributed in several tissues, unless indicated (*), whereby Pearson's testing was performed, and the correlation coefficients are shown.

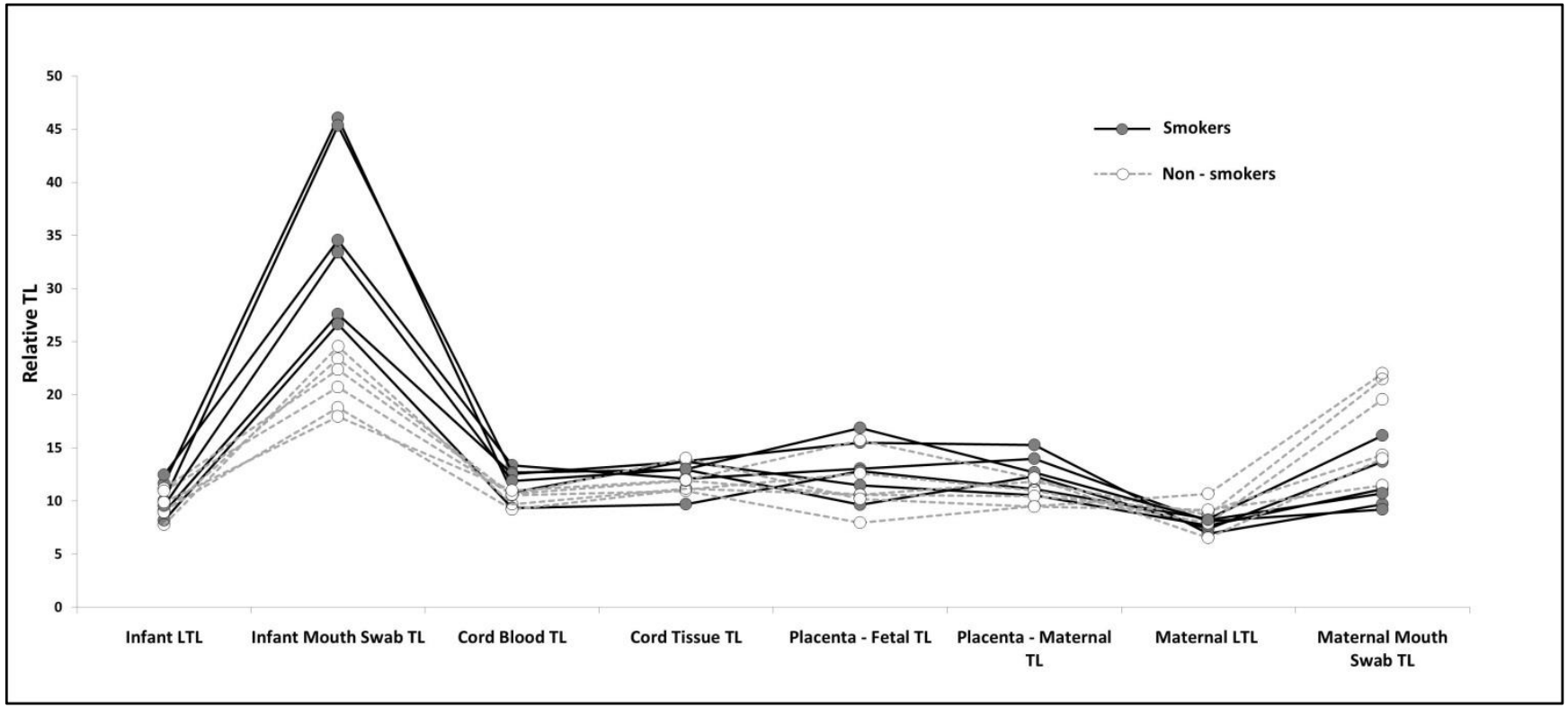

Figure 3.9. Intra-individual pattern of TL across different tissues (n =12, 6 smokers and 6 non-smokers).

3.4 Discussion

The rate of smoking in our study sample was higher than that observed in the Canadian general population but consistent with the relatively low socio-economic status of the women enrolled in the cohorts. The reliability of smoking self-report during pregnancy within our cohorts was shown to be highly concordant with plasma cotinine concentrations.

In this small sample, we consistently observed univariate associations between maternal smoking during pregnancy and longer TL at birth in several infant tissues including leukocytes, mouth epithelial cells, and umbilical cord leukocytes. This effect remained after controlling for infant sex. Sensitivity analyses showed that this was not related to ethnic imbalance, nor the prevalence of preterm birth between smoking and non-smoking groups. There were no statistically significant independent associations between maternal smoking during pregnancy and TL in cord blood and cord tissue, for which sample size was very limited. However, the observed effect sizes were of similar magnitude as that seen for LTL, suggesting non-negligible effects in these tissues as well.

These results are counterintuitive, as numerous studies have consistently reported shorter TL in persons who smoke, an effect believed to be related to increased oxidative stress and cellular turnover. Given this, a similar effect might have been expected in fetuses exposed to maternal smoking. However, our findings are in agreement with those recently published [260], showing longer CD4+ T cell TL in infants born to smoking mothers. The authors suggested a possible relationship with cytogenetic cell damage brought about by *in utero* exposure to maternal smoking [260]. The mechanism behind longer TL in smoking exposed infants remains

unclear, and our study was not designed to address this. It is known that carcinogenic metabolites of nicotine can cross the placenta [300] and consequently, exposure to cigarette smoke could result in DNA alterations and chromosome instability in the fetus [301,302]. This could in turn result in lowered cellular replication rates, and thereby maintain TL. A few studies have reported decreased number of lymphocytes and neutrophils in neonates exposed to maternal tobacco smoking *in utero* [303–305]. It is possible that smoking induced *in utero* stresses may predispose leukocytes with shorter telomeres to be preferentially eliminated during fetal development, resulting in residual cell populations with an apparent longer TL.

Our findings of lower GA and birth weight in smoking-exposed infants is in agreement with other studies [282,283], and could reflect both down-modulation of cellular replication *in utero* and/or smoking-induced pregnancy stresses. Notably, we observed no association between infant TL and small for GA. The fact that smoking during pregnancy seems to affect TL in a wide variety of infant tissues could suggest that the effect is systemic, and not restricted to leukocytes. However, it could also indicate that intra-individual TL is correlated between tissues. We observed a possible association between preterm birth and longer maternal TL which is intriguing and merits further investigation. Given the small sample and limited number of preterm events overall, this finding could be spurious. Unlike previously reported [292], our exploratory analyses failed to show independent associations between maternal smoking and shorter maternal TL, whether in leukocytes or mouth epithelial cells. However, that study involved a large sample of women with a broad age range. In our smaller study, smoking was more prevalent among younger women, and the participants' age was limited to the narrower reproductive age range, possibly masking any effect.

We also observed a significant association between preterm birth and longer placental TL, consistent with a previous report [306]. This result is intuitively plausible since delivery before full term of gestation may be accompanied with fewer cell divisions and/or restricted growth of the organ, resulting in less attrition, hence longer telomeres.

As in numerous other studies, we saw an increased incidence of preterm birth and lower birth weight associated with maternal smoking during pregnancy. Such continued evidence associating adverse pregnancy outcomes with smoking highlights the need for smoking cessation interventions, especially among women of reproductive age.

3.5 Strengths and Limitations

A major strength is that we present the first detailed study investigating the effects of exposure to maternal smoking during pregnancy on TL in a variety of infant and maternal tissues. Limitations include the heterogeneity in smoking data collection across the three cohorts, forcing us to define this variable categorically as smoking ever during pregnancy (yes *vs.* no). However, based on the subset of women for whom more extensive data was available, the majority of women who smoked at any time in pregnancy continued smoking throughout their pregnancy.

Paternal age, known to influence progeny TL, was unavailable. Given that smoking mothers were younger, the fathers of exposed infants were likely also younger, a factor associated with shorter infant TL [264,307]. Paternal age is therefore unlikely to confound the association of longer TL seen here. Ethnicity has also been linked to LTL and could have

confounded some results. Our sensitivity analyses among the subset of infants born to white mothers helped address this issue but paternal ethnicity data was not available.

Another limitation is the small sample size for some tissues, as those were not collected in all cohorts. This reduced our power and limited our ability to control for weakly associated factors. Finally, the study was not *a priori* designed to investigate infant LTL in relation to maternal smoking and should be prospectively reproduced.

3.6 Conclusions

In conclusion, we found that maternal smoking during pregnancy was independently associated with longer infant birth LTL. Although in other tissues the associations did not reach statistical significance, a similar effect was observed. This counterintuitive effect may be a result of slower telomere attrition if tobacco exposure affects fetal cell proliferation/differentiation, or preferential elimination of fetal cells with short telomeres. It could also be an apparent effect resulting from smoking stress modulating the survival of progenitor cells with longer telomeres. These observations could be relevant to future epidemiological studies investigating the predictive value of infant telomere length on health outcomes.

Chapter 4: BLOOD mtDNA LEVELS ARE PERSISTENTLY ELEVATED FROM BIRTH INTO EARLY LIFE IN cART-EXPOSED HIV-EXPOSED UNINFECTED CHILDREN

4.1 Abbreviated introduction to chapter

The objective of this study was to compare blood mtDNA content at birth in a large cohort of HEU and HUU children, and investigate possible relationships between mtDNA content and *in utero* exposure to maternal cART. I hypothesized that mtDNA content would be elevated at birth and may be associated with certain maternal cART regimens. I also report on the longitudinal changes in mtDNA content over the first three years of life among HEU children.

4.2 Methods

4.2.1 Study population

The study population consisted of the same cohort of HEU and HUU children <3 years of age, for whom LTL at birth and during early life is detailed in chapter two (refer to Figure 2.1). A subset of children in this study was previously examined [251] and included again here, to ensure a wide breadth of *in utero* ARV exposure with respect to type and duration of cART. Importantly, all their specimens were re-assayed using the MMqPCR technique. Infant and maternal demographic and clinical data, including exposures during pregnancy were obtained from the cohort databases as described in chapter two, section 2.2.1.

4.2.2 MtDNA content measurement

WB mtDNA content was expressed as a ratio between mtDNA and nuclear DNA copies, measured via monochrome multiplex qPCR as previously described [308]. We assayed all specimens from an individual HEU participant on the same plate to minimize variability. Plates contained both HEU and HUU specimens, randomized. The intra and inter-assay variability were 7.9% and 8.4%, respectively. We excluded specimens that did not meet the quality control criteria of <15% difference between replicates over two assay attempts [308].

4.2.3 Statistical analyses

All mtDNA content values were $\log_{10}$ transformed to achieve normal (HUU) or near normal (HEU) distributions. Clinical and demographic characteristics of HEU and HUU study participants were compared using the Student's t or Mann-Whitney U tests for continuous variables, and the χ^2 test for categorical variables. Spearman's correlations were used to investigate univariate associations between $\log_{10}$ mtDNA content at birth and GA at birth, birth weight, maternal age at delivery, and duration of *in utero* cART exposure. We used t-tests or Mann-Whitney U tests to assess associations between mtDNA content at birth and the following: HIV exposure status (HEU *vs.* HUU), infant sex, preterm birth, SGA, and smoking ever during pregnancy. We also compared mtDNA content at birth between various ethnicities and cART regimens using Kruskal-Wallis or ANOVA tests, with Dunn's or Tukey's tests to adjust for multiple pairwise comparisons. Factors important univariately ($p<0.10$) were considered for inclusion in multivariable ANCOVAs using backward stepwise selection. Collinearity between variables was assessed and if present, variables were included in the model in turn.

To determine the relationship between WB mtDNA content and age during the first three years of life, a subset of distinct HEU and HUU children were sex- and age-matched 1:1 (± 2 days within the first 2 weeks; ± 8 days from 2 weeks to 1 year; ± 15 days from 1-3 years). We then compared the slopes and intercepts of the two groups' linear regressions using GraphPad Prism V7. We also used generalized additive mixed-effects models or linear mixed-effects models to analyze the longitudinal trajectory of mtDNA content among all HEUs with the 'mgcv' package in R v.3.4.2. Finally, comparisons of mtDNA content between birth and the closest subsequent visit during the prophylaxis period used paired t-tests.

4.3 Results

4.3.1 Characteristics of study participants

4.3.1.1 All participants

A total of 324 HEU children had one or more WB specimens available for this study, collected between birth and three years of age. Of these, 114 (35%) had a birth specimen, and 214 (66%) had ≥ 2 longitudinal specimens. For HUU children, 306 had a single WB specimen each, 88 (29%) of these were collected at birth, and 154 (50%) were anonymous controls. Infant and maternal characteristics, including exposures during pregnancy, are detailed in Chapter two, section 2.3.1, Table 2.2A. Briefly, among HEU and HUU children for whom this information was available, maternal age, history of smoking during pregnancy, infant sex, GA at birth, birth weight, preterm status, and SGA were similar. However, they differed significantly in maternal ethnicity, with ~50% of HEU children compared to <1% of HUU children having been born to African Caribbean Black women ($p<0.001$).

Seven (2%) HEU children were not exposed to cART *in utero* and were excluded from analyses at birth. Among the remaining 317 HEUs, 124 (38%) were born to mothers who initiated HIV therapy prior to conception, 39 (12%) during the first trimester (0-13 weeks), 124 (38%) during the second trimester (>13-26 weeks), and 29 (9%) during the third trimester. The median (IQR) duration of *in utero* cART exposure was 24.4 (16.6 - 37.4) weeks. Most HEUs were exposed to a combination of AZT+3TC (n=221, 69%), with either LPV/r (n=112, 35%), NFV (n=83, 36%), or NVP (n=26, 8%). Thirty two (10%) were exposed to ABC+3TC+PI/r, 27 (8%) to TDF+FTC or 3TC+PI/r, and the remaining 36 (11%) were exposed to other cART regimens (detailed in Table S1). All HEU children received AZT prophylaxis for a median (IQR) of 6 (5 – 6) weeks.

4.3.1.2 Participants with a birth specimen

Demographics for the 114 HEU and 88 HUU children with a birth specimen (0-3d) are presented in Chapter two, section 2.3.1, Table 2.2A. In addition to the imbalance in ethnicity whereby mothers of HEU children were more often Indigenous (29%) or African Caribbean Black (25%) compared to HUU children (8% and 2%, respectively), HEU infants had lower GA and weight at birth (both $p<0.001$). All other characteristics were similar between groups, although rates of maternal smoking during pregnancy were higher (55% in the HEU group and 43% in the HUU group) than the corresponding all participant groups (Table 1A). Once again, HEU children were mostly exposed to AZT+3TC+LPV/r (39%) or AZT+3TC+NFV (30%) *in utero*, with a median (IQR) exposure of 20.6 (14.8-36.6) weeks.

4.3.2 Infant mtDNA content at birth

MtDNA content at birth was analyzed in 114 HEU and 86 HUU children, as measurements for two HUU failed assay quality control. In univariate analyses, HEU infants had significantly higher mtDNA content at birth compared to HUU infants (Figure 4.1A). Lower GA (Figure S2, Table 4.1) and prematurity (Figure 4.1B, Table 4.1) were significantly associated with higher mtDNA content at birth in both HEU and HUU children. Lower infant birth weight was significantly associated with higher mtDNA content overall, but this was mostly influenced by the HEU group (Table 4.1). Maternal smoking during pregnancy was also associated with higher mtDNA content at birth overall, but this was primarily influenced by the HUU group (Figure 4.1C, Table 4.1). A significant interaction between HEU/HUU status and maternal smoking ($p=0.04$) was detected, and was included in the multivariable models. Lastly, infant sex and maternal ethnicity were not associated with mtDNA content at birth (Table 4.1). However, HEUs exposed to AZT+3TC+LPV/r ($p=0.017$) or ABC+3TC+PI/r ($p=0.02$) *in utero* had significantly higher mtDNA content at birth compared to HUU controls, although some groups were small (Figure 4.1D).

Within the HEU group, duration of *in utero* cART exposure was not associated with mtDNA content at birth, when analyzed either as weeks of exposure during gestation or trimester of maternal cART initiation (Table 4.1). Additionally, there were no differences in HEU mtDNA content at birth between cART types.

Table 4.1. Univariate analyses of the association between possible predictors and infant mtDNA content at birth

Explanatory variables	All infants (n = 200)		HEUs (n = 114)		HUUs (n = 86)	
	r_s [a]	p value	r_s	p value	r_s	p value
HIV exposure status (HEU vs. HUU)	---	**0.001**	---	---	---	---
Infant Sex, Male (ref. Female)	---	0.89	---	0.65	---	0.31[c]
GA (per week)	-0.281	**<0.001**	-0.241	**0.01**	-0.213	**0.001**
Preterm Birth (ref. Term birth)	---	**0.001**	---	**0.04**	---	**0.006**
Birth Weight (per kg)	-0.278	**<0.001**	-0.256	**0.006**	-0.167	0.12
SGA, Yes (ref. No)	---	0.09	---	0.25	---	0.27[c]
Maternal age (per year)	-0.086	0.22	0.039	0.68	-0.180	0.10
Maternal ethnicity:						
Indigenous	---	0.14[b]	---	0.31[b]	---	**0.03[d]**
African Caribbean Black						
White						
Asian and Others						
Maternal smoking ever in pregnancy, Yes (ref. No)	---	**0.007**	---	0.55	---	**0.001[c]**
HIV pVL (>50 copies/ml) close to delivery, Detectable (ref. undetectable)	---	----	---	0.69	---	---

Explanatory variables	All infants (n = 200)		HEUs (n = 114)		HUUs (n = 86)	
	r_s [a]	p value	r_s	p value	r_s	p value
Duration of *in utero* cART exposure (per week)	---	---	-0.06	0.52	---	---
Maternal cART started:						
Before conception	---	---	---	0.94[b]	---	---
1st trimester						
2nd trimester						
3rd trimester						
Maternal cART regimen:						
AZT+3TC+NVP	---	---	---	0.16[b]	---	---
AZT+3TC+NFV						
AZT+3TC+LPV/r						
ABC+3TC+PI/r						
TDF+FTC(or 3TC)+PI/r						

[a]Spearman's rho, [b]Kruskal-Wallis p value, [c]Student's t-test p value, [d]ANOVA with Tukey's test p value (Asian vs. Indigenous Tukey's adjusted p=0.03)

Abbreviations: HEU – HIV-exposed uninfected, HUU – HIV-unexposed uninfected, GA – Gestational Age, SGA – Small for Gestational Age, cART – combination Antiretroviral Therapy, AZT – Zidovudine, 3TC – Lamivudine, NVP – Nevirapine, NFV – Nelfinavir, LPVr – Ritonavir-boosted Lopinavir, ABC – Abacavir, PI/r – Ritonavir-boosted Protease Inhibitor, TDF – Tenofovir Disoproxil Fumarate, FTC - Emtricitabine

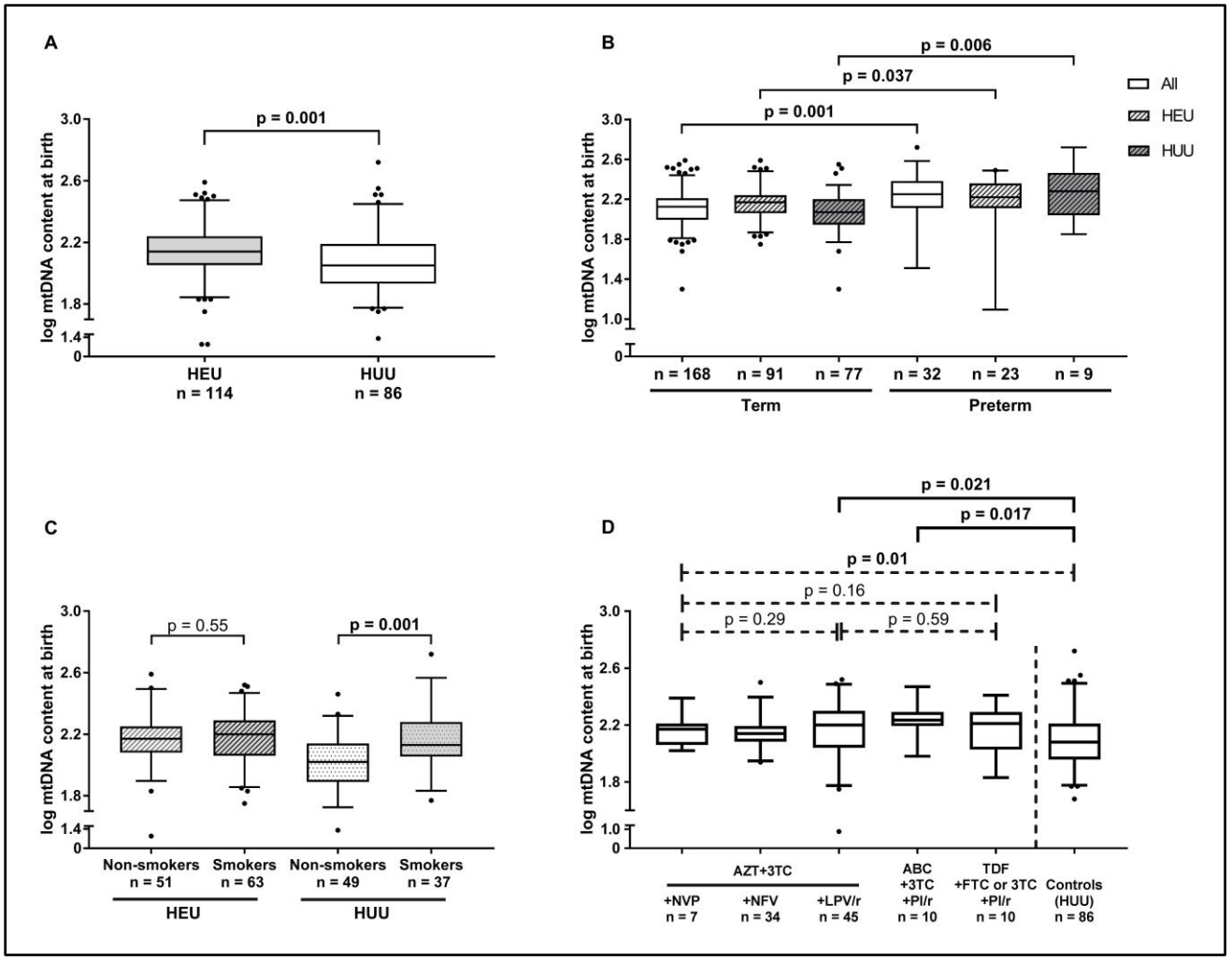

Figure 4.1. Unadjusted comparisons of mtDNA content at birth. A – HEU and HUU infants (Mann-Whitney U test). B – infants born at term and preterm (Mann-Whitney U test). C –HEUs and HUUs exposed to maternal smoking during pregnancy or not (Mann-Whitney U test). D – HEUs exposed to different cART regimen in utero and HUUs [dashed brackets indicate Kruskal Wallis p value while solid brackets indicate Dunn's adjusted p value (adjusted for multiple comparisons)]. For all panels, whiskers of the box plots represent the 5-95 percentiles.

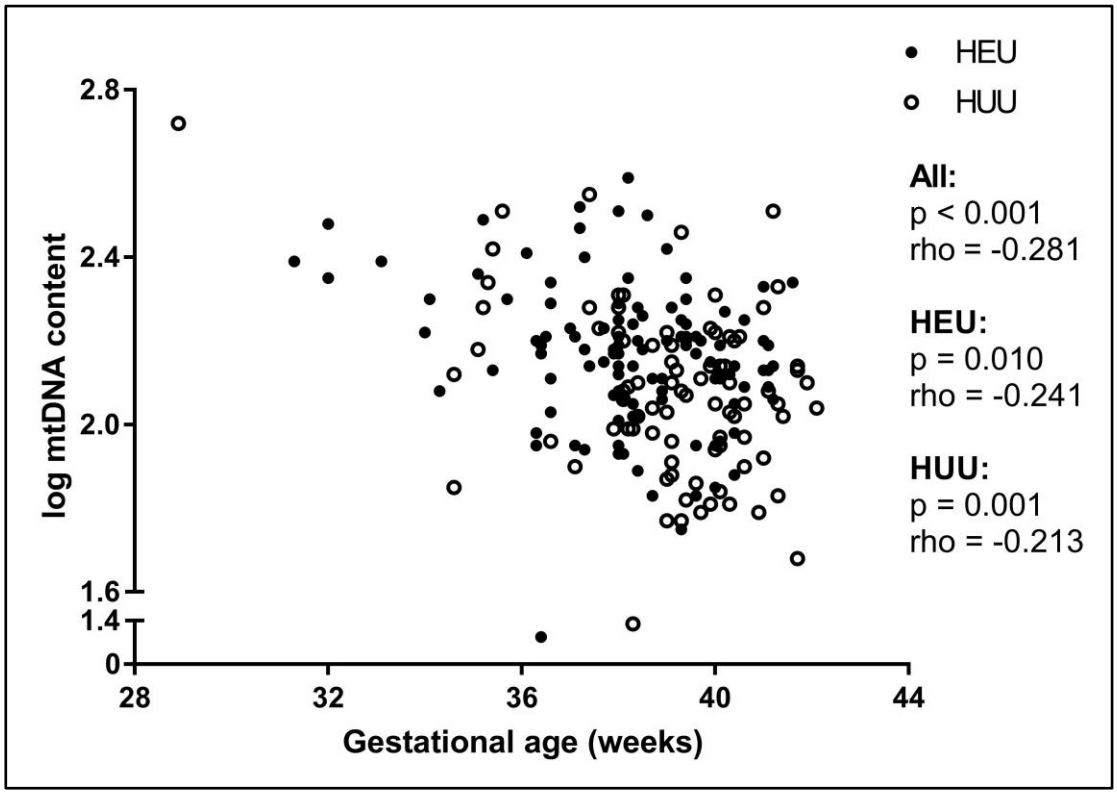

Figure 4.2. Spearman's correlation between mtDNA content at birth and gestational age among all participants, as well as HEU and HUU groups separately.

In the final multivariable model of all participants (n=192, Figure 4.3A) that included HEU/HUU status, GA at birth, maternal smoking, and the HEU/HUU status*maternal smoking interaction term, lower GA at birth was independently associated with higher mtDNA content at birth. This model further suggests that HEU status and being born to mothers who smoked were also associated with higher mtDNA content. However, this must be interpreted with caution given the behaviour of the interaction term which, together with the HEU and HUU models (Figure 4.3C-D), clearly imply that the association between smoking and mtDNA is restricted to the HUU children who show low mtDNA content when born to non-smoking mothers (Figure 4.1C). HEUs grouped by cART exposure (Figure 4.3B) had similar mtDNA content at birth as HUUs.

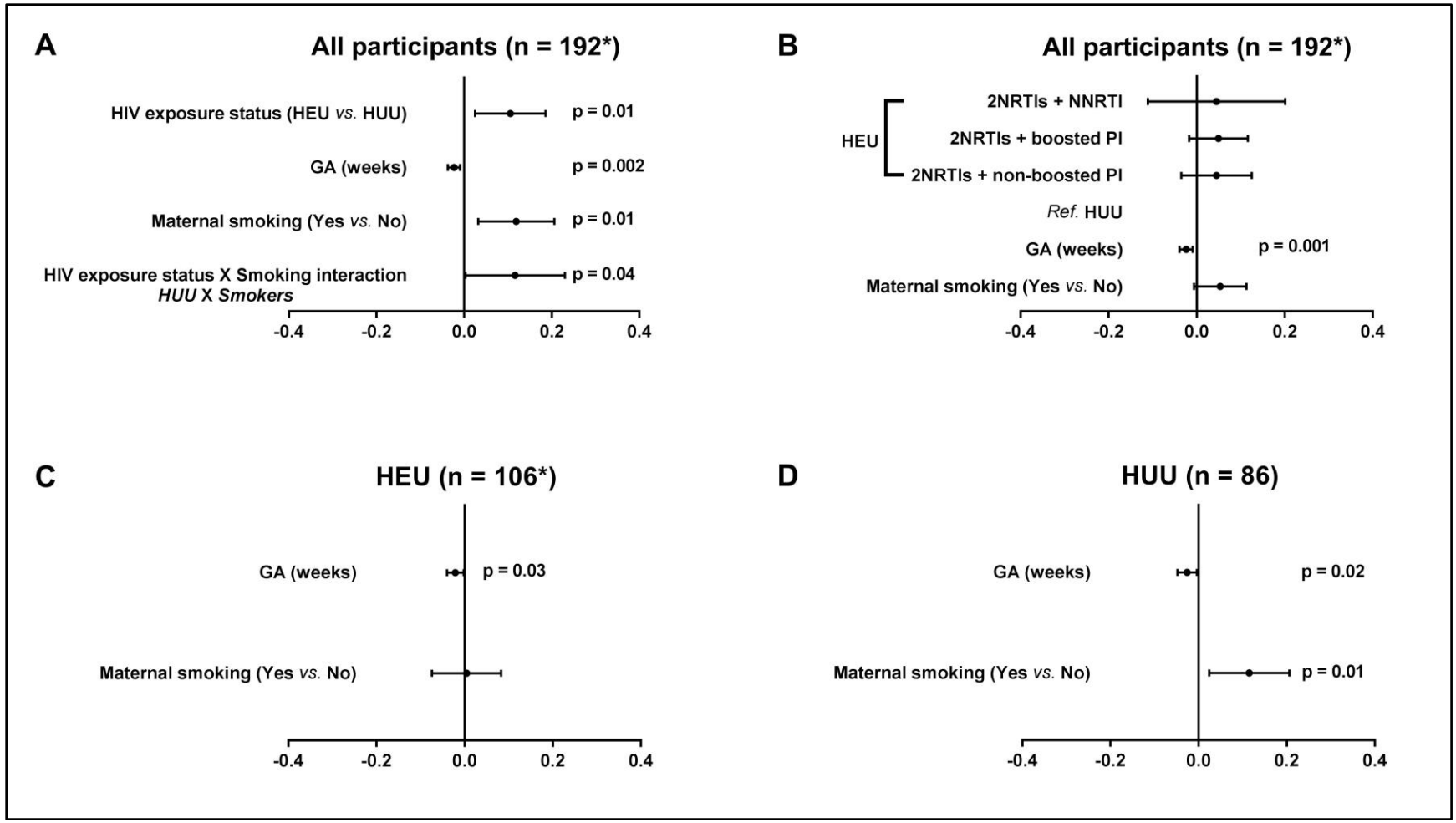

Figure 4.3. Multivariable regression analyses of the association between possible predictors and infant mtDNA content at birth.
A – all participants (n=192, R^2 = 0.14). B – all participants with HEUs separated by type of cART exposure in utero (n=192, R^2 = 0.12). C – HEUs (n=106, R^2 = 0.05). D – HUUs (n=86, R^2 = 0.18). *8 HEUs who were exposed to non-standard cART regimen in utero were excluded from these analyses

Given the imbalance in ethnicity between the two study groups, a sensitivity analysis that included only children born to White mothers (n=93; 39 HEU, 54 HUU) was performed. This model was similar to that of the full sample, suggesting that ethnicity was not an important confounder (Figure 4.4).

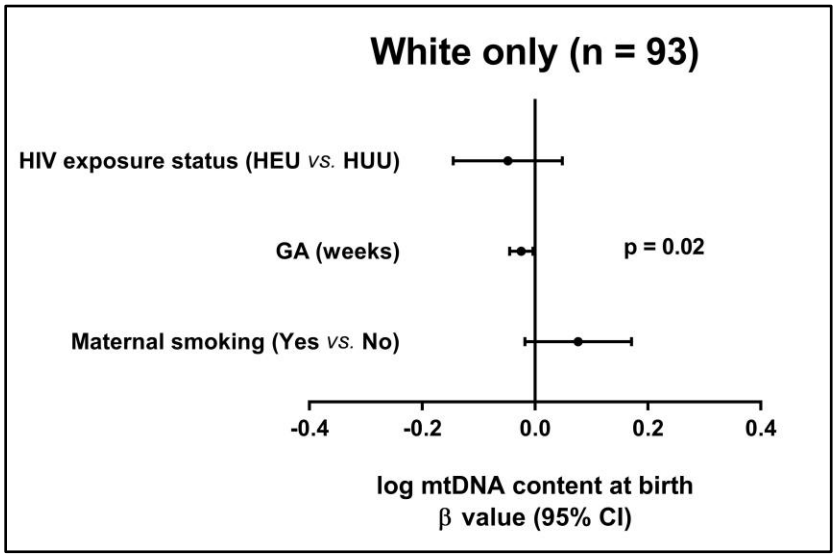

Figure 4.4. Multivariable regression analysis among all White participants (sensitivity analysis) showing the association between possible predictors and infant mtDNA content at birth (n=93, R^2 = 0.10). Effect sizes are expressed as non-standardized β values.

Furthermore, given the strong relationship between prematurity and higher mtDNA content at birth, and because few HUUs were preterm, a second sensitivity analysis that included only children born at term (n=161, HEU=84, HUU=77) was performed. The results were similar to the full sample, with a weakened association with GA (Figure 4.5A). Upon subdividing HEUs according to type of *in utero* cART exposure, only infants exposed to 2NRTIs + boosted-PI showed significantly higher mtDNA content at birth compared to HUU children (Figures 4.5B). Further inspection revealed that this effect was restricted to infants exposed to boosted-PI on a backbone of AZT+3TC or ABC+3TC (Figure 4.6).

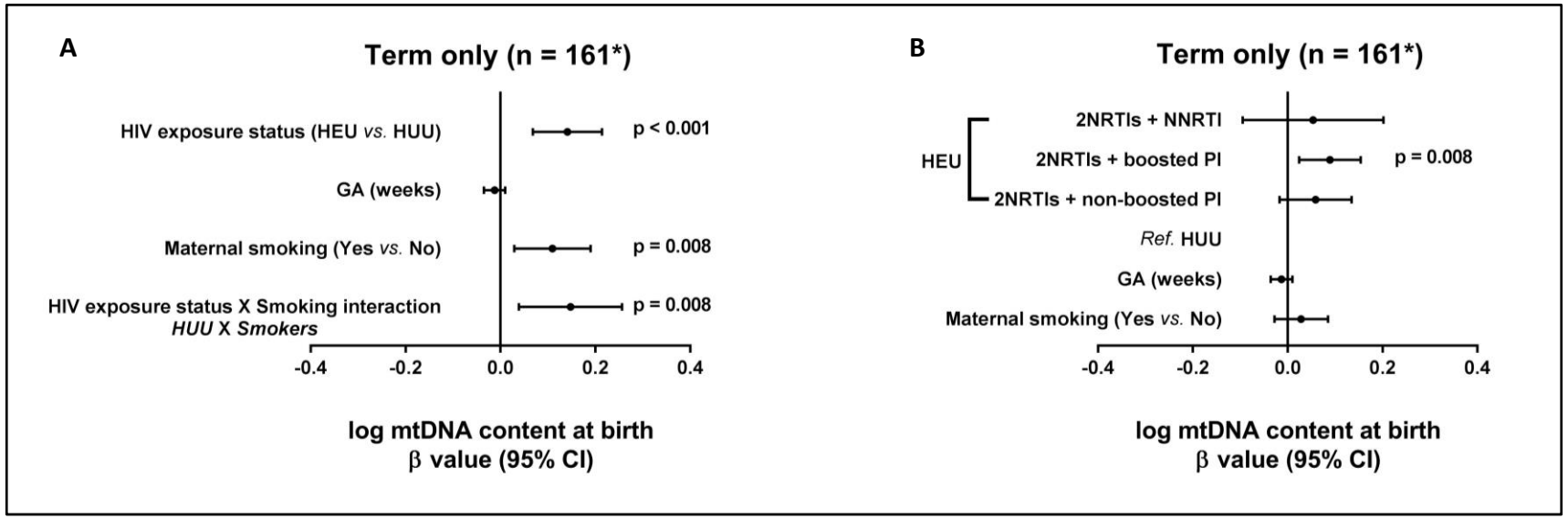

Figure 4.5. Multivariable regression analyses of the association between possible predictors and infant mtDNA content at birth among infants born at term (sensitivity analysis). A – all term (n=161, R^2 = 0.12), and B – all term with HEUs separated by type of cART exposure in utero (n=161, R^2 = 0.08). Effect sizes are expressed as non-standardized β values.

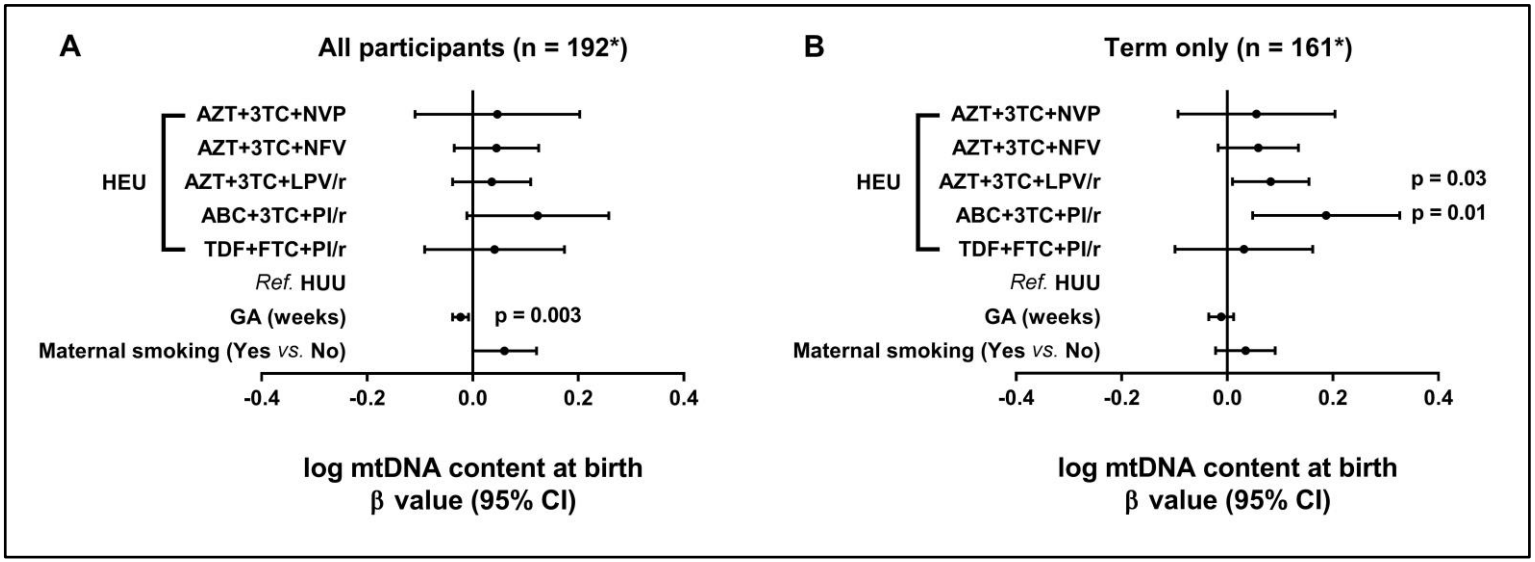

Figure 4.6. Multivariable regression analyses of the association between possible predictors and infant mtDNA content at birth. A – all participants with HEUs separated by detailed type of cART exposure in utero (n=192, R^2 = 0.13). B – all term only with HEUs separated by detailed type of cART exposure in utero (n=161, R^2 = 0.10). Effect sizes are expressed as non-standardized β values.

4.3.3 Cross-sectional comparison of mtDNA content during the first three years of life

For this analysis, 214 distinct HEU children were age- and sex-matched 1:1 with 214 HUU children, as discussed previously in Chapter two, section 2.3.3, Table 2.5. The two groups showed nearly identical slopes for the linear regressions of mtDNA content *vs.* age (Figure 4.7). However, the intercepts (elevation) showed that HEU children have significantly higher mtDNA content on average than HUU children throughout the first three years of life ($p<0.001$, Figure 4.7). The results were unchanged in sensitivity analyses excluding children pairs containing either a preterm child (Figure 4.8A), or a child exposed to maternal smoking (Figure 4.8B).

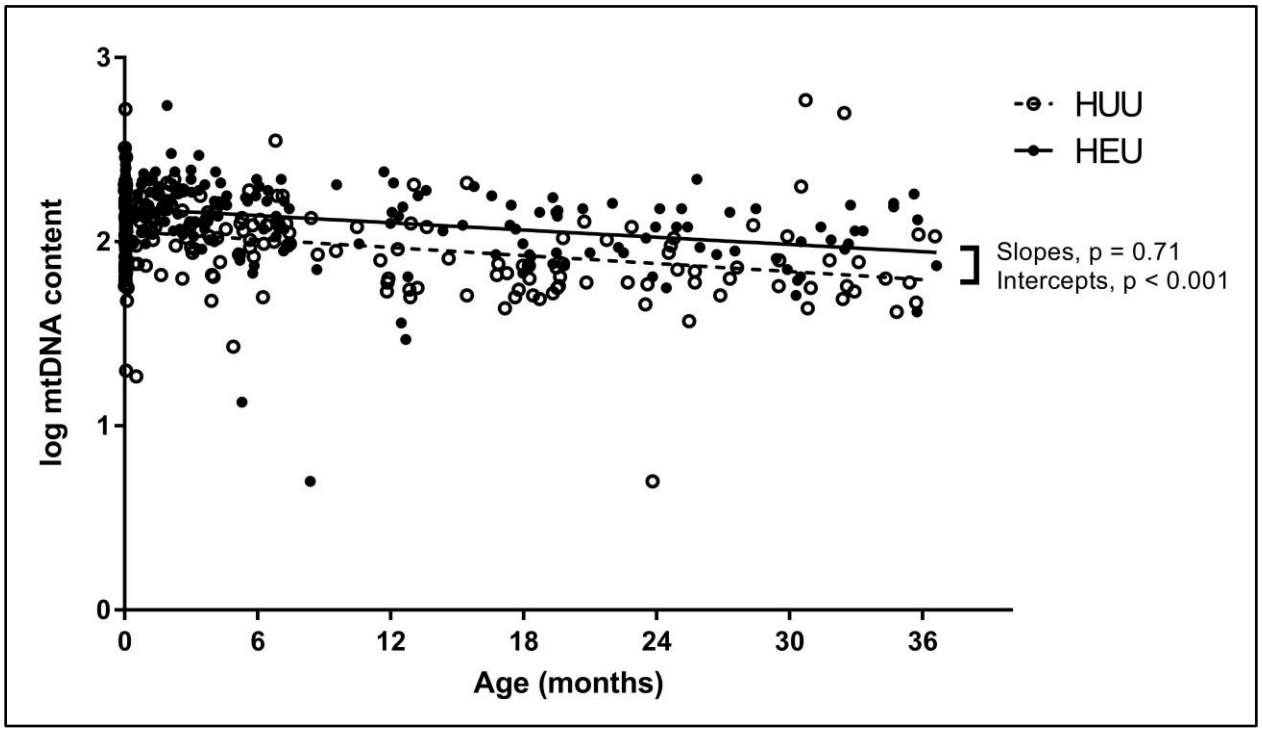

Figure 4.7. Relationship between mtDNA content and age during the first three years of life among age- and sex-matched children.

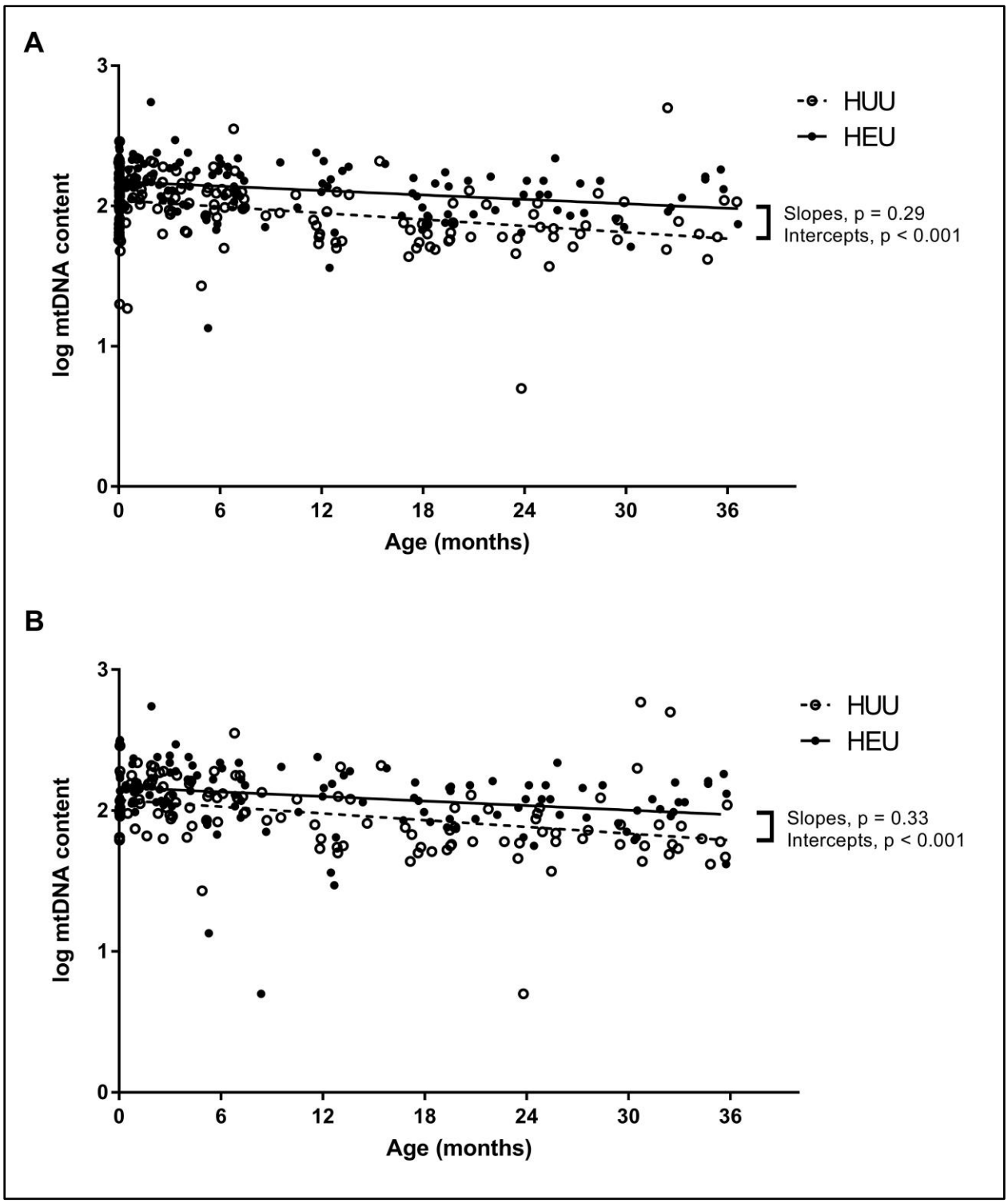

Figure 4.8. Sensitivity analysis showing the relationship between mtDNA content and age during the first three years of life among age- and sex-matched children: A – restricted to term children (n=155 matched pairs). B – restricted to children born to women who did not smoke during pregnancy (n=125 matched pairs).

A similar cross-sectional analysis, whereby HEU and HUU mtDNA content were grouped into different 'age bins' according to their age at blood sampling, yielded consistent results (Figure 4.9).

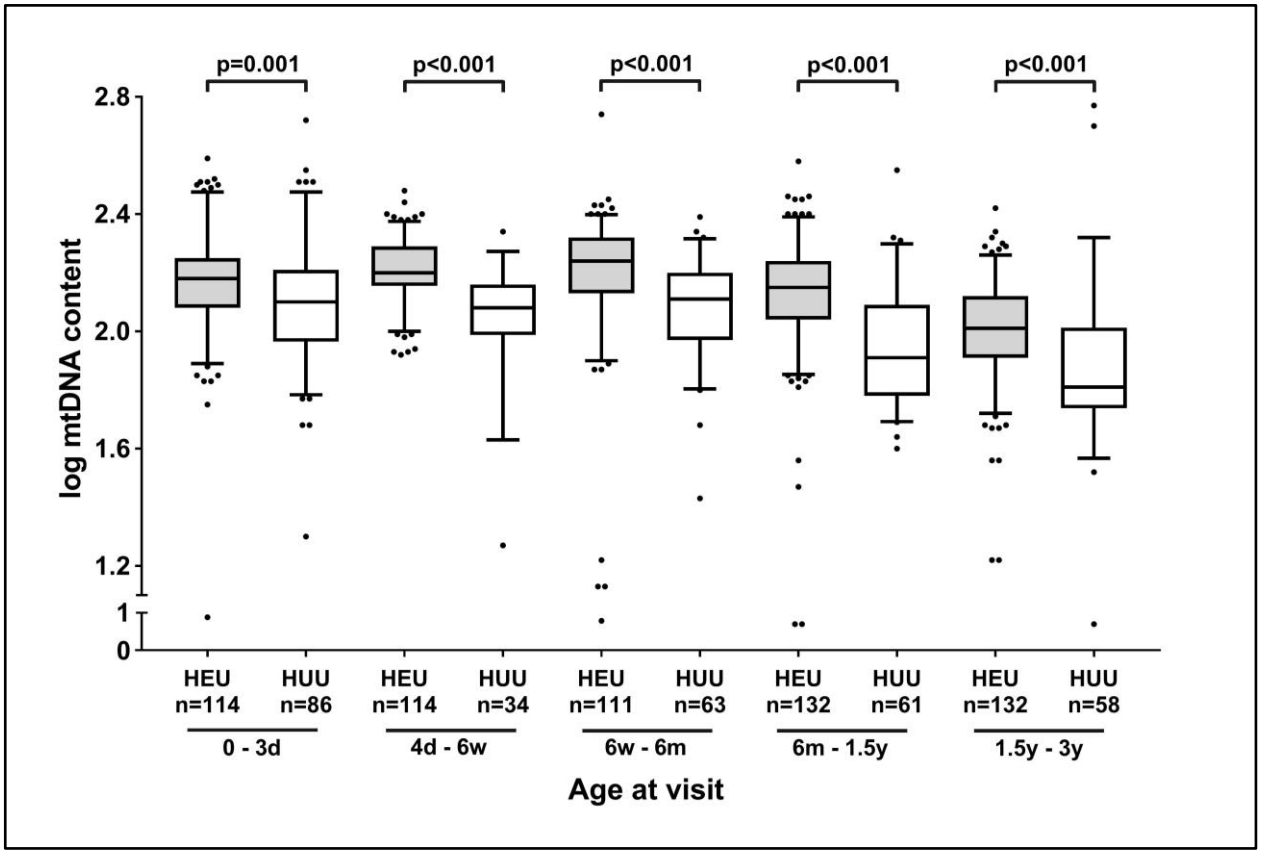

Figure 4.9. Comparison of all HEU and HUU mtDNA content divided into five age bins: 0-3d (birth), 4d-6w (approximate AZT prophylaxis period), 6w-6m, 6m-1.5y and 1.5y-3y. Solid brackets indicate Mann-Whitney U test p values. Whiskers represent the 5-95 percentiles.

4.3.4 Longitudinal mtDNA content dynamics among HEU children

Longitudinal changes in mtDNA content over the first three years of life were assessed for the 214 HEU children for whom ≥2 blood specimens were available. A significant non-linear relationship between mtDNA content and age was observed, with mtDNA content remaining relatively unchanged during the first six months of life, followed by a gradual decrease up to age two, and a leveling out thereafter (Figure 4.10). The fit of the additive mixed-effects model was only marginally better than that of a linear mixed-effects model (likelihood-ratio test p=0.03). The latter suggested a gradual decrease in mtDNA content over time (p<0.001, Figure 4.11), at a rate (approximately -20/year) similar to that seen for the HUU group's linear regression (single specimen per participant, p<0.001, Figure 4.11).

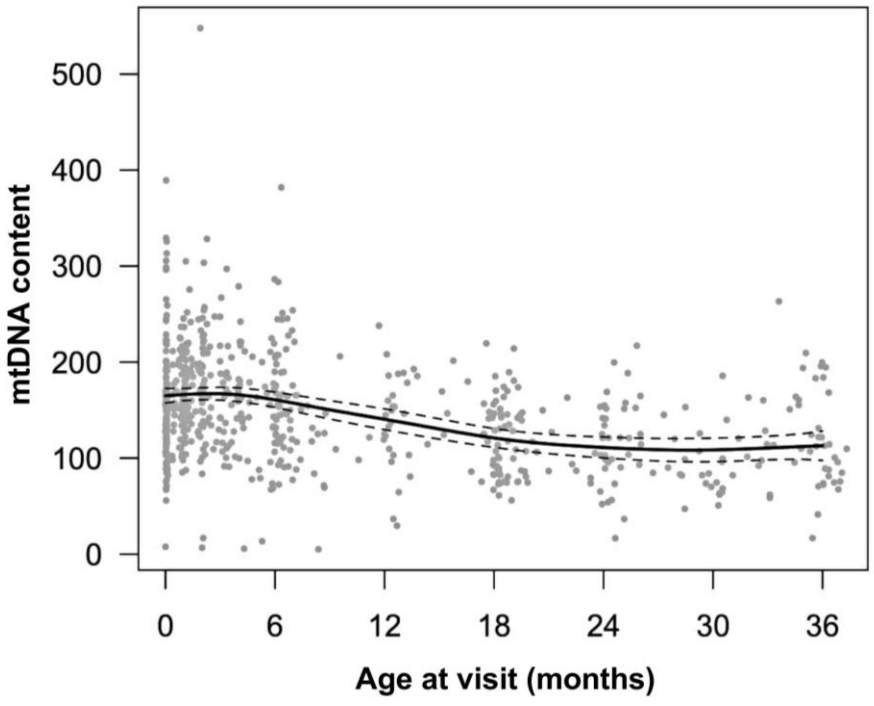

Figure 4.10. Non-linear regression model (generalized mixed effects additive model) of mtDNA content and age (in months) for HEU infants.

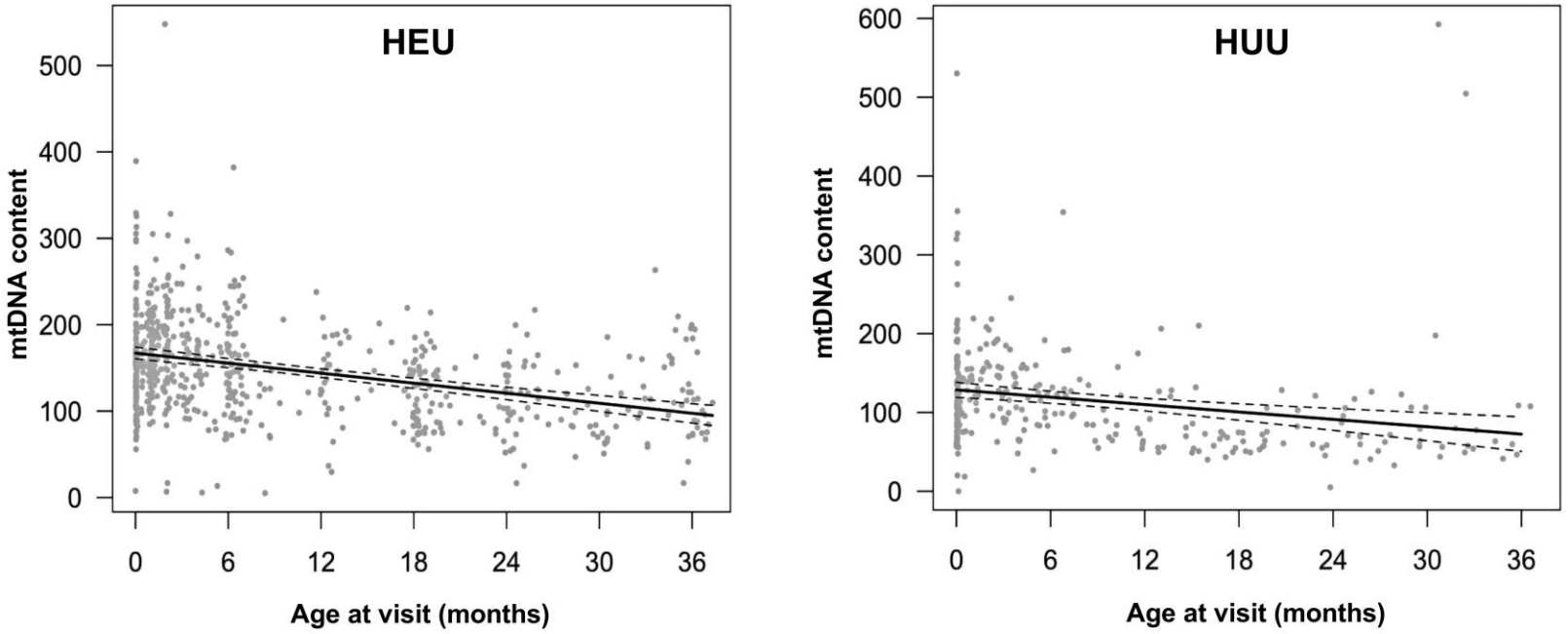

Figure 4.11. Linear regression model (generalized mixed effects linear model) of mtDNA content and age (in months) for HEU and HUU infants.

4.3.5 HEU mtDNA content during prophylaxis

Given the observed association between type of *in utero* cART exposure and mtDNA content at birth, we investigated whether six weeks postnatal AZT prophylaxis affected mtDNA content using paired HEU specimen. There was no difference in mtDNA content between birth and the closest subsequent visit during prophylaxis which was at a median [range] age of 31 [18-47] days (n=58, p=0.11, Figure 4.12).

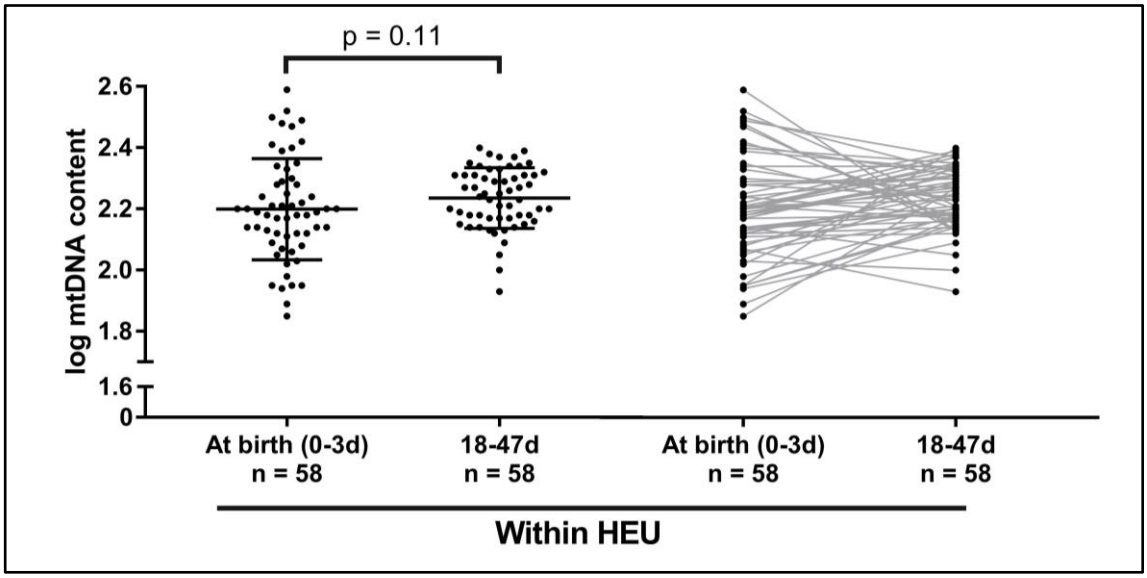

Figure 4.12. Comparisons of mtDNA content at birth and the subsequent visit (18-47d) among HEU children (paired Student's t test).

4.4 Discussion

Consistent with previous studies by our group [251,254] and others [252,253], we observed an association between HIV/cART exposure *in utero* and elevated blood mtDNA content at birth compared to HUU controls, an effect that persisted throughout the first three years of life. Importantly, only HEUs exposed to ritonavir-boosted PI regimens had higher mtDNA content at birth compared to HUUs; we observed no associations with exposures to NRTIs [93,111]. It is noteworthy that a majority of the participants in two other US studies reporting higher HEU mtDNA content were also exposed to PI-containing maternal regimens [252,253]. Although the mechanism of this effect is unclear, *in vitro* studies have shown that PIs increase ROS and can cause oxidative stress, disrupt mitochondrial intermembrane potential, and increase cellular apoptosis [309]. All these may plausibly result in altered mtDNA levels. Potential mechanisms include adaptive mitochondrial biogenesis, altered proportions of immune cell subsets with different mtDNA content, or a dysregulation of mitochondrial replication/mitophagy homeostasis in response to *in utero* HIV/cART-associated stresses. Our study was not designed to address putative mechanism(s).

Our findings are in contrast with older studies that reported lower mtDNA levels in HEU children [244,246–249]. However, HEUs exposed to ritonavir-boosted PIs were scarce in these studies, with most pregnant women receiving either single or dual AZT-containing regimens. Others were exposed to older NRTIs such as stavudine and didanosine, both known to decrease mtDNA [111,218,310,311]. Most of these studies acknowledge limitations related to sample size and/or heterogeneity in the recruitment of study and control participants, a possible source of

bias. Additionally, heterogeneity in the type of specimens studied may partially explain discrepancies between studies.

We observed an independent association between lower GA and higher peripheral blood mtDNA content at birth; however, this effect was primarily driven by preterm children born before 37 weeks of gestation. Another group reported a similar negative correlation between umbilical cord blood mtDNA content and GA [312]. A recent study reported higher cord blood mtDNA content in the context of placental insufficiency characterized by preeclampsia and/or intrauterine growth restriction, but not in association with preterm birth *per se*. However, their study sample contained only eight preterm births, selected for their lack of preeclampsia and/or intrauterine growth restriction, which may have limited power and/or induced bias [313]. We are not aware of any other published study investigating infant mtDNA in preterm birth.

Several studies report an increased risk of preterm birth among HEU exposed to PI-based regimens *in utero* [192–198]; others report no such association [201–203]. In our study, *in utero* exposure to boosted-PI regimens was associated with elevated mtDNA content at birth, with no evidence of an association between preterm birth and type of cART regimen. However, we had limited power to detect such association(s) if present and our data do not link maternal HIV/cART, preterm birth and mtDNA content. It is possible that mtDNA content naturally declines toward the end of the gestational period, and this could explain our observation of higher mtDNA content among preterm children. However, given the association of higher mtDNA with smoking, it could be linked to a stress response related to being born preterm.

Studies in animal models or larger prospective cohorts of blood and other tissues would be required to investigate this.

Of note, cART-exposed HEU children have a >2-fold higher risk of being born preterm compared to HUU children [192,193,314,315], something we observed in our cohort among children with a birth specimen. However, the similar preterm birth rates observed within the full sample, ~20% in both groups, suggests an apparent overrepresentation of preterm children in our HUU group. This may be related to biases associated with participant recruitment. HIV-negative controls were a convenience sample recruited at hospital/clinics during routine visits to their pediatricians; it is possible that children born preterm would have more frequent visits, favouring their recruitment. Alternatively, as we made a deliberate effort to enroll control children with similar sociodemographic characteristics as the HEU group, HUUs may have had inherently higher preterm birth risk compared to the general population.

Within the HUU group, our finding of higher mtDNA content at birth in association with maternal smoking during pregnancy is consistent with another report in cord blood serum of children exposed to tobacco smoke prenatally [316]. This may be related to adaptive mitochondrial biogenesis in response to smoking-induced oxidative stress, an effect possibly masked by exposure to HIV/cART in the HEU group.

Finally, our longitudinal analysis revealed that HEU mtDNA content remains relatively unchanged very early in life, and gradually decreases thereafter. The implications of higher mtDNA content at birth and in early life remains unclear, given that both lower and higher

mtDNA content have been associated with cancers, disease, fertility, organ development, and ageing in the general population [85]. Further investigations are required to determine the clinical meaning of harbouring higher mtDNA content and whether this is associated with health outcomes later in life.

4.5 Strengths and Limitations

Ours is the largest longitudinal study of mtDNA content in HEU children during the first three years of life. However, we lacked longitudinal specimens from HUU children, and this precluded us from conducting detailed comparisons between groups. Platelets contain mtDNA and information on platelet count was unavailable. However, disorders related to low platelet count such as thrombocytopenia may be exacerbated by HIV/cART exposure, and would present a conservative bias. Furthermore, we observed no correlation between platelet count and mtDNA content in a previous study of older HEU children [254]. Our analyses were conducted on WB mtDNA, and as such, our measurements reflect mtDNA in both cells and in plasma and cannot tease the two apart. Circulating or cell-free mtDNA is a powerful damage associated molecular pattern that leads to immune activation and induction of an inflammatory response. Among adult and pediatric individuals, we previously reported independent associations between elevated cell-free mtDNA and both HIV infection and younger age [317]. In the same study, we were unable to compare HEU and HUU participants, as we had a limited number of young HUU children. Therefore, in addition to WB mtDNA, future studies quantifying the levels of cell-free mtDNA in HEU and HUU children may help provide a more complete picture of the aging process in these children. Our cohort also had some demographic and behavioural imbalances. For example, nearly half the HEU children but <1% of HUU children were African Caribbean

Black. However, ethnicity was not an important predictor of mtDNA content at birth, as suggested by our sensitivity analysis restricted to White children. Rates of maternal smoking during pregnancy were high for both the HEU and HUU groups. Given that the association between maternal smoking during pregnancy and higher mtDNA content at birth was observed only among HUU children, this may be related to differences in smoking behaviour (frequency, intensity and duration) between groups. Based on our previous study [298], the majority of HIV-positive and HIV-negative women who reported smoking at any time during their pregnancy continued doing so throughout, with very few quitting. Finally, we lacked information on maternal HIV plasma viral load, CD4 count, comorbidities, co-infections including HCV and CMV, illicit substance use, and other maternal exposures that may have confounded our results.

4.6 Conclusions

In conclusion, higher mtDNA content at birth was associated with exposures to stressors such as maternal HIV, maternal smoking, and boosted-PI cART, or being born preterm, itself possibly related to stresses. This may represent a rebound following removal of said stressor(s) when the child is born. These results merit further investigations on the long-term mitochondrial effects of HIV/cART exposure and the potential implications of altered mtDNA content as a predictor of health in this expanding population of HIV-uninfected, cART-exposed individuals.

Chapter 5: PHARMACOLOGICAL CONCENTRATIONS OF cART AFFECT MITOCHONDRIAL AND CELLULAR HEALTH IN A TRANSFORMED CELL CULTURE MODEL: PROTECTIVE ROLE OF HUMAN TELOMERASE REVERSE TRANSCRIPTASE

5.1 Introduction

Recent advances in HIV treatment have seen the integration of the InSTIs DTG and RAL into first-line cART regimens. InSTIs are potent ARVs that induce rapid viral suppression, including in pregnant women presenting with HIV late in pregnancy, as well as adolescents and pediatric patients [26–29]. Further, there has been a steady shift from TDF- to TAF-based NRTI therapy; the latter showing reduced insults on kidney and bone health [318–320]. Given the ever-changing landscape of HIV treatment strategies, drug toxicity becomes a major concern. It is crucial to investigate the short- and long-term effects of these newer drugs, to ensure not only efficacy but long term safety.

Aside from mtDNA depletion, several human and *in vitro* studies have linked older antiretrovirals of the NRTI, NNRTI and PI classes with alterations in mitochondrial morphology, mitochondrial mass, OXPHOS enzyme activities, cellular autophagy, and apoptosis, indicative of mitochondrial dysfunction [102,138,155,309,321–324]. Currently used InSTIs such as RAL and DTG are known to have excellent clinical tolerance, with no major cases of toxicity [33,320,325]. A few recent studies have reported higher rates of neuropsychiatric adverse events with the use of DTG compared to RAL, leading to discontinuation of treatment in these

individuals [32–34]. However, most of these studies were limited to small number of patients, and this may have contributed to an overestimation of DTG-associated adverse events. Importantly, a recent study reported increased incidence of neural-tube defects among Botswana children exposed to maternal DTG since conception [35], while a group from the United Kingdom reported no such neural-tube defects with the use of RAL or EVG in pregnancy [326]. An *in vitro* study using human hepatoblastoma cells and primary rat neurons also reported no evidence of mitochondrial toxicity, characterized by changes in mitochondrial intermembrane potential (MMP) and superoxide levels, and/or reduced cell viability, following 24 h treatment with pharmacological concentrations of RAL [327]. However, data regarding the effects of TAF and InSTIs on mitochondrial function are extremely limited, and the short- and long-term health effects of these newer ARVs remain unclear.

To address this gap, the present study primarily aimed to investigate changes in cellular and mitochondrial health following exposure to current and clinically relevant cART regimens at pharmacologically relevant concentrations. Previous work in our lab has shown that exposure to certain cART regimens can affect mitochondrial morphology, mtDNA content and cellular proliferation in placental and lymphoblast cell lines [328]. I conducted a similar, but more extensive investigation of cART-associated toxicities using a transformed human lung fibroblast cell line model transduced to express components of human telomerase. As detailed previously in chapter 1, hTERT has extra-telomeric roles, including translocating to the mitochondria to protect the organelle and its DNA from oxidative damage [161,162]. The presence of hTERT in the mitochondria has been associated with decreased ROS generation, increased MMP and reduced cellular apoptosis [159–161,163]. Therefore, a secondary objective of this study was to

determine whether cART-associated toxicities, if present, would be modulated by mitochondrial hTERT. As hTERT is only expressed in some tissues such as stem cells and placenta, this possible modulation of toxicities could be relevant.

The cell line used in this study, SV40 transformed WI-38 primary-fibroblast-derived VA 13 subtype, utilizes the alternate lengthening of telomere (ALT) mechanism to maintain telomeres, a non-physiological homologous recombination-based system [329][330]. This parental ALT cell line was engineered by our collaborator to express components of human telomerase via retroviral transduction, in order to help address our research questions. Since the overall theme of my thesis centers around the effects of HIV/cART during pregnancy and markers of cellular health, most regimens investigated in this study were in line with current treatment guidelines for pregnant women.

5.2 Materials and Methods

5.2.1 Reagents and drugs

Dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) pH 7.4 were purchased from Sigma-Aldrich (Oakville, ON, Canada). Cell culture reagents such as Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and 0.25% Trypsin-EDTA (1X) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and Gibco (Life Technologies, Eugene, OR, USA). Culture flasks and multi-well plates were purchased from Corning Life Sciences (Falcon®, Tewksbury, MA, USA). All ARVs were obtained from the National Institutes of Health (NIH) AIDS Reagent Program and reconstituted in 100% DMSO. Working stocks of ARVs were prepared to ensure a final concentration of 0.1% DMSO in culture. Table 5.1 shows the maximum plasma concentration (C_{max}) of ARVs that were used in all experiments.

Table 5.1. Maximum plasma concentration (Cmax) of ARVs used in this study.

	NRTIs						NNRTI	PIs				InSTI	
ARVs	**ABC**	**AZT**	**3TC**	**FTC**	**TDF**	**TAF**	**EFV**	**NFV**	**LPV**	**DRV**	**RTV**	**RAL**	**DTG**
C_{max}* (μM)	4.47	8.57	6.54	7.28	0.52	0.47	12.90	6.03	15.59	9.17	1.23	6.53	8.32

* Values are obtained from the United States Food and Drug Administration (FDA) https://www.accessdata.fda.gov

5.2.2 Selection of cART regimen

The cART regimens investigated in this study were based on those recommended by North American and WHO guidelines for treatment of pregnant women LWH, as described in chapter 1, section 1.4.1, Table 1.2 [172–178]. Briefly, these included a combination of previously recommended first-line pregnancy regimen, as well as regimen that are expected to be used more extensively during pregnancy in the future. Current WHO guidelines no longer recommend the use of AZT+3TC+LPV/r, and instead favour the use of TDF+FTC+EFV as first-line therapy to treat adults and pregnant women LWH. Aside from TDF+FTC, the other preferred NRTI backbone is ABC+3TC. Both backbones are used in combination with PIs such as LPV/r or DRV/r. Canadian guidelines for the treatment of pregnant women adhere to a combination of two NRTIs and a PI/r due to proven safety[179]. The success of DTG in controlling HIV pVL has resulted in the formulation of a once daily, single-tablet regimen ABC+3TC+DTG (brand name – Triumeq®), that was approved by the FDA in August 2014 and is being increasingly used nowadays. However, the short- and long-term safety of InSTIs during pregnancy, both for the mother and the developing fetus, remain unclear, and were therefore included in our evaluation.

Taken together, I initially chose to study the following cART regimens for my short-term experiments: AZT+3TC ± LPV/r or NFV, previously recommended and among the most frequently used cART during pregnancy, including in North America; TDF+FTC ± EFV or DTG, two currently advocated first-line regimens for the treatment of adults and pregnant women; ABC+3TC ± DTG, an alternative first-line regimen; and TAF+FTC ± EFV or DTG, to compare TDF- *vs.* TAF-containing tenofovir backbones. Since all ARVs were dissolved in

DMSO for a final concentration of 0.1% DMSO in culture, a drug vehicle control of 0.1% DMSO was included as a negative control.

For my longitudinal experiments, cART regimens were revised, following the recent findings of 'a potential early signal for increased prevalence of neural-tube defects in association with exposure to DTG-based cART from the time of conception among children from Botswana' [35]. This prompted the inclusion of RAL, to allow comparisons between the effects of DTG vs. RAL. Following consultation with infectious diseases obstetricians in Vancouver regarding current PI/r-based regimen for the treatment of pregnant women in Canada, the now sparingly used backbone of AZT+3TC was excluded. Instead, the ABC+3TC backbone was expanded to include DRV/r and LPV/r. The final cART regimens studied include: TDF+FTC+EFV, TDF+FTC+DTG, TDF+FTC+RAL, TAF+FTC+DTG, ABC+3TC+DRVr, ABC+3TC+LPV/r and ABC+3TC+DTG. The 0.1% DMSO negative control was included in triplicates, to minimize variability for data normalization.

5.2.3 Cell culture

Three isogenic sublines of the ALT cell line, WI-38 VA-13 subline 2RA (ATCC), were obtained from our collaborator Dr. Judy Wong (Associate Professor, Faculty of Pharmaceutical Sciences, UBC). These included: 1) VA-13 parental cell control expressing empty retroviral vectors (no telomerase components); 2) VA-13 expressing wildtype (WT) hTERT capable of translocating to the mitochondria, and the human telomerase RNA component (hTR); and 3) VA-13 expressing hTR and recombinant hTERT lacking the mitochondrial targeting signal (MTS), thereby incapable of mitochondrial translocation [331]. The study was therefore

designed to investigate whether mitochondrial protection by hTERT is present, and if so, is it modulated by its ability to translocate to the organelle. The three isogenic ALT cell lines will be herein referred to as –hTERT, +hTERT and +hTERT –MTS, respectively. They were cultured in DMEM + 10% FBS medium, according to ATCC recommendations, with 5 µg/mL Puromycin (Selleckchem, Burlington, ON, Canada) and 50 µg/mL Hygromycin (Invitrogen, Thermo Fisher Scientific, Waltham, MA, US) antibiotic selection, as the retroviral vectors included genes conferring resistance to these antibiotics. All experiments were performed at 37°C in a humidified atmosphere of 5% CO_2.

For the short-term cART exposure experiments, the three ALT cell lines were seeded in 6-well plates at 200,000 cells per well and incubated for ~6 hours to allow cell attachment. Following this, media was removed from each well and fresh media containing 1X C_{max} of cART regimens at 0.1% final DMSO concentration was added, and incubated. After three days, the medium was removed and cells were exposed to 200 µL of trypsin EDTA solution for 5-8 minutes at 37°C (until cells detached) and collected for various assays. A total of five independent experiments were conducted, each with a single well per treatment and control.

For the longitudinal experiments, a pilot experiment conducted prior to this ascertained that seeding directly into cART-containing medium did not affect cell attachment, cell viability or cell proliferation (data not shown). Therefore, ALT cells were seeded as above, but directly into media containing 1X C_{max} of cART regimens or 0.1% DMSO (negative control). Cells were exposed to cART regimens for nine days, following which they were returned to cART-free medium (0.1% DMSO) for six days, to allow recovery and mimic a cART interruption. During

this entire time, cells were sub-cultured/passaged every three days by removing media and trypsinizing as before. Fresh cART-containing media (treatment phase) or 0.1% DMSO media (recovery phase) was added to each well at each passage. Cell viability by trypan blue exclusion staining and cell counts of the resultant suspensions were determined at each passage using a Bio-Rad TC-10^{TM} automated cell counter (Bio-Rad, Mississauga, ON, Canada). These measures were used to seed (200,000 cells/well) for the next time points. The remaining cells (500,000 or less) were processed for DNA extractions at baseline (day 0), days 3, 6, 9 (during the treatment phase), and days 12 and 15 (during the recovery phase) by centrifugation for 6 mins at 2000 rpm, followed by resuspension in lysis buffer (AL, Qiagen, Toronto, ON, Canada) and freezing at -80°C. Samples for flow cytometry were obtained at day 9 (end of treatment) and day 15 (end of recovery). All experiments were repeated five times independently.

5.2.4 DNA extraction and mtDNA content measurement

Total genomic DNA was extracted using the 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 a minor modification: cell pellets collected for DNA extraction were initially re-suspended in AL buffer and hence, the latter was replaced with PBS during the extraction protocol. MtDNA content was measured using the same MMqPCR assay described in Chapter 4 for days 3, 6 and 9 of the treatment phase, and day 15 at the end of the recovery phase. For data presentation and analyses, mtDNA content for each treatment was normalized to that of the corresponding 0.1% DMSO control for each cell line.

5.2.5 Flow cytometry

Flow cytometry was conducted on no less than 2 x 10^4 and no more than 1 x 10^5 cells per treatment from each experiment, using a BD LSR II-561 analyzer (BD Biosciences, Mississauga, ON, Canada) at the UBC FLOW Core. Cell viability was ascertained using the fluorescent DNA-binding dye 4′,6-diamidino-2-phenylindole (DAPI) (BD Biosciences, Mississauga, ON, Canada), while cellular apoptosis was quantified using BV711-conjugated Annexin V (BD Biosciences, Mississauga, ON, Canada). Cells treated with 500 nM Staurosporine for 24h were used as a positive control for Annexin V staining. Mitochondrial parameters were assessed on live cells only, based on viability gating. Mitochondrial mass, MMP, and mitochondrial ROS were quantified using the mitochondrial probes MitoTracker Green FM, MitoTracker Deep Red FM and MitoSOX Red, respectively (Thermo Fisher Scientific, Waltham, MA, US). All probes/dyes were individually titrated, following which, cells were stained according to recommended protocols. Analysis of flow cytometry data used the FlowJo software v10.4.2. For data analyses, Median Fluorescence Intensity (MFI) values were normalized to the MFI value of the corresponding 0.1% DMSO control.

5.2.6 Statistical considerations

Statistical analyses were restricted to the longitudinal experiments. For the purposes of this thesis, only interim analyses on select comparisons were performed and these are described in subsequent sections. Additional experiments will be performed in the coming months and the data will be re-analyzed for the manuscript. Comparisons of specific cART treatment groups to the corresponding DMSO control were done on raw data via Mann Whitney U tests. The latter was also used to compare differences between cART groups within a cell line, as well as cross-

sectionally between different cell lines, on data normalized to DMSO controls. Relationships between mitochondrial parameters were examined using Spearman's correlation. Data are presented as median with IQR.

5.3 Results

5.3.1 Short-term cART experiments

The 3-day experiments were primarily designed to guide subsequent longitudinal experiments, and identify whether treatment with 1X C_{max} of cART regimen for this short period would elicit alterations in cellular and mitochondrial health.

All three ALT cell lines demonstrated similar responses to cART exposure, with no overt differences in overall cell viability (Figure 5.1). The +hTERT and +hTERT –MTS ALT cells appeared to be more tolerant to cART pressure, with a lowest median cell viability of 92% and 93%, respectively across all cART regimen. For the –hTERT cells, the lowest median cell viability observed was 66% for the TAF+FTC backbone. Five out of the 11 remaining treatments had median % cell viability between 83-92%, with three of these being DTG-based regimen.

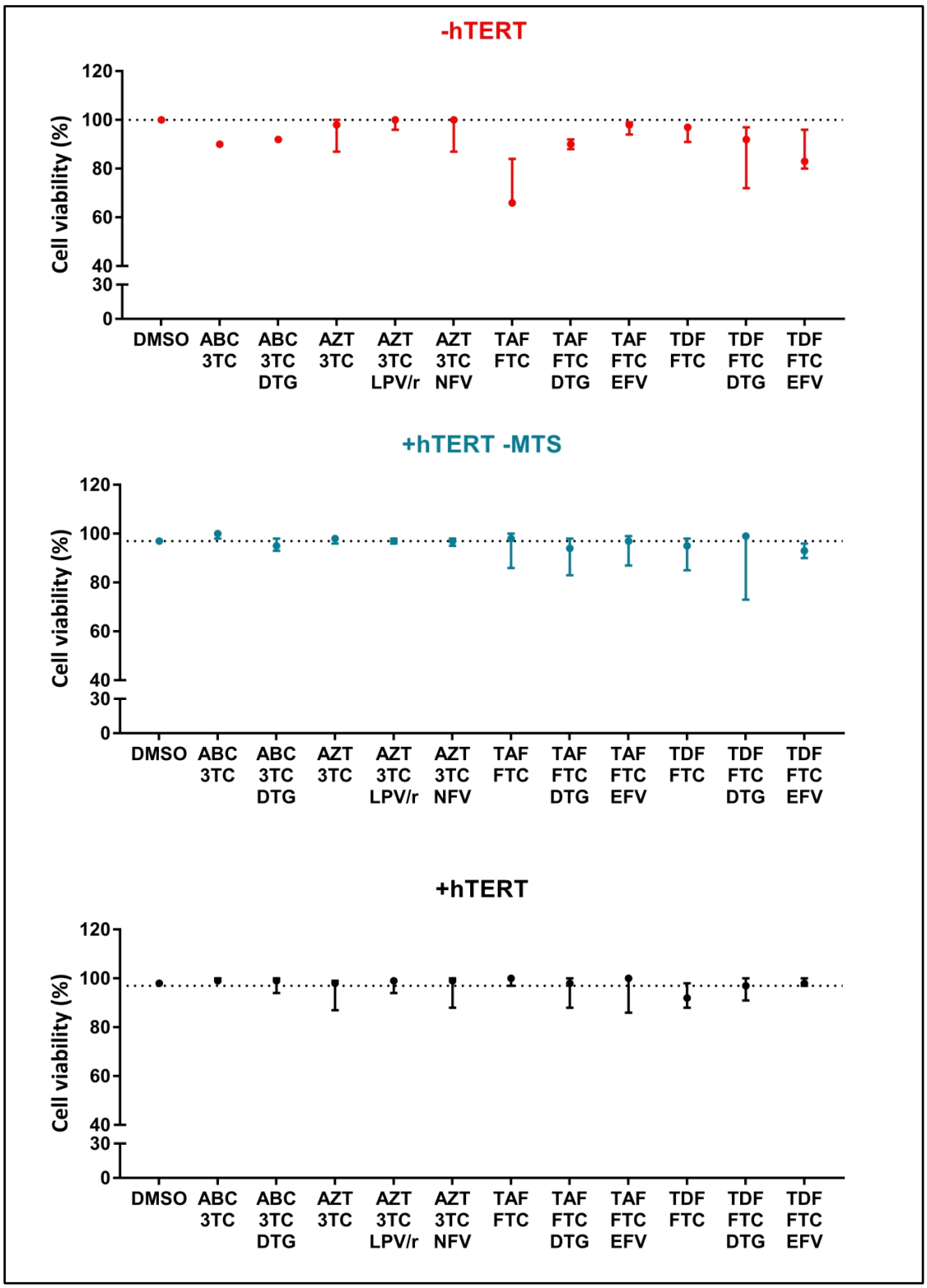

Figure 5.1. Effects of three-day exposure to 1X Cmax cART on cell viability among ALT cell lines. Data are median with IQR, with horizontal line representing the median % cell viability of cells treated with 0.1% DMSO (for comparison).

Mitochondrial mass, MMP and ROS were not drastically different between cART regimen, and across all three ALT cells (Figures 5.2 and 5.3). A visual inspection of the data suggests a moderate increase in mitochondrial mass for –hTERT cells treated with AZT+3TC+LPV/r, TAF+FTC, TAF+FTC+DTG and TAF+FTC+EFV. Mitochondrial mass was also moderately elevated for +hTERT –MTS cells treated with TDF+FTC+EFV, whereas no noticeable differences were seen for the +hTERT cells. On the other hand, MMP appeared to be elevated for most cART regimen in the –hTERT and +hTERT –MTS cell lines, but this was less evident for the +hTERT cell line. Interestingly, both DTG- and EFV-based regimen on backbones of TDF+FTC or TAF+FTC appeared to have higher MMP across all three cell lines, with the median increase in MMP ranging from 21% (+hTERT cell line) to almost 100% (-hTERT cell line). Further, the TDF+FTC and TAF+FTC backbones themselves also seem to increase MMP, compared to the DMSO controls and the other AZT+3TC and ABC+3TC backbones.

Finally, although cART did not affect ROS levels considerably, a trend was observed whereby TDF+FTC+DTG and TAF+FTC+DTG regimen across all three ALT cell lines had slightly elevated ROS, ranging from a median 21% to 35% increase. Interestingly, the TDF+FTC and TAF+FTC backbones alone did not seem to affect ROS levels, suggesting that the observed increase may be due to the addition of the DTG base. Furthermore, the effects of the two DTG-containing regimens were comparable to increased ROS levels observed with exposure to PI-based cART regimen (LPV/r and NFV) across cell lines (median 25% to 29% increase).

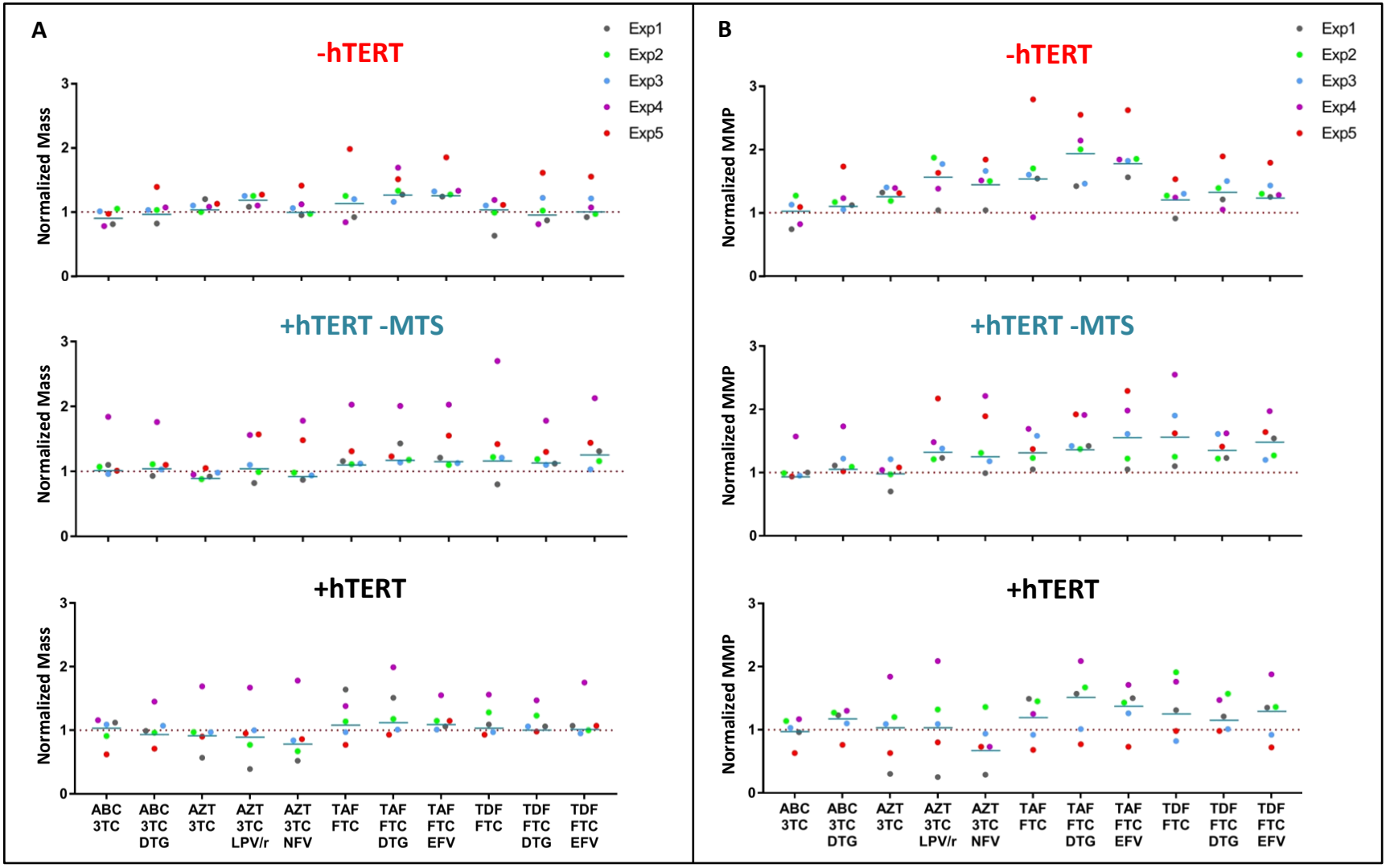

Figure 5.2. Effects of three-day exposure to 1X Cmax cART on (A) mitochondrial mass, and (B) mitochondrial membrane potential (MMP) among ALT cells. Data (n=5 independent experiments) were normalized to control values (cells treated with 0.1% DMSO, represented by the horizontal line at 1). Short horizontal blue lines represent the median of each data set.

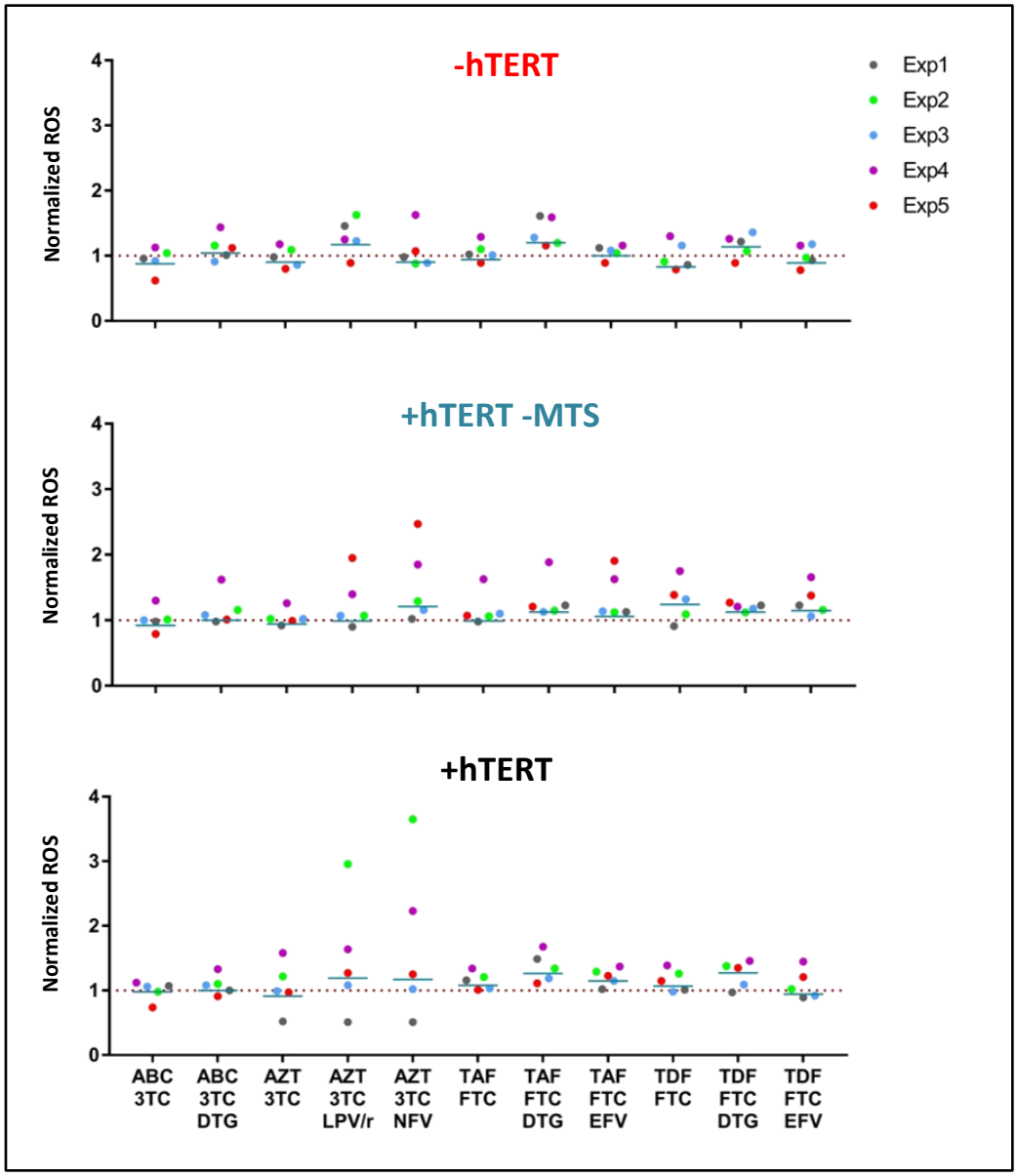

Figure 5.3. Effects of three-day exposure to 1X Cmax cART on mitochondrial ROS among ALT cells. Data (n=5 independent experiments) were normalized to control values (cells treated with 0.1% DMSO, represented by the horizontal line at 1). Short horizontal blue lines represent the median of each data set.

MtDNA content was also fairly similar across cART regimen and cell lines (Figure 5.4). However, the +hTERT cell line appeared to have generally higher mtDNA content compared to the corresponding cART regimen in the two other ALT cell lines.

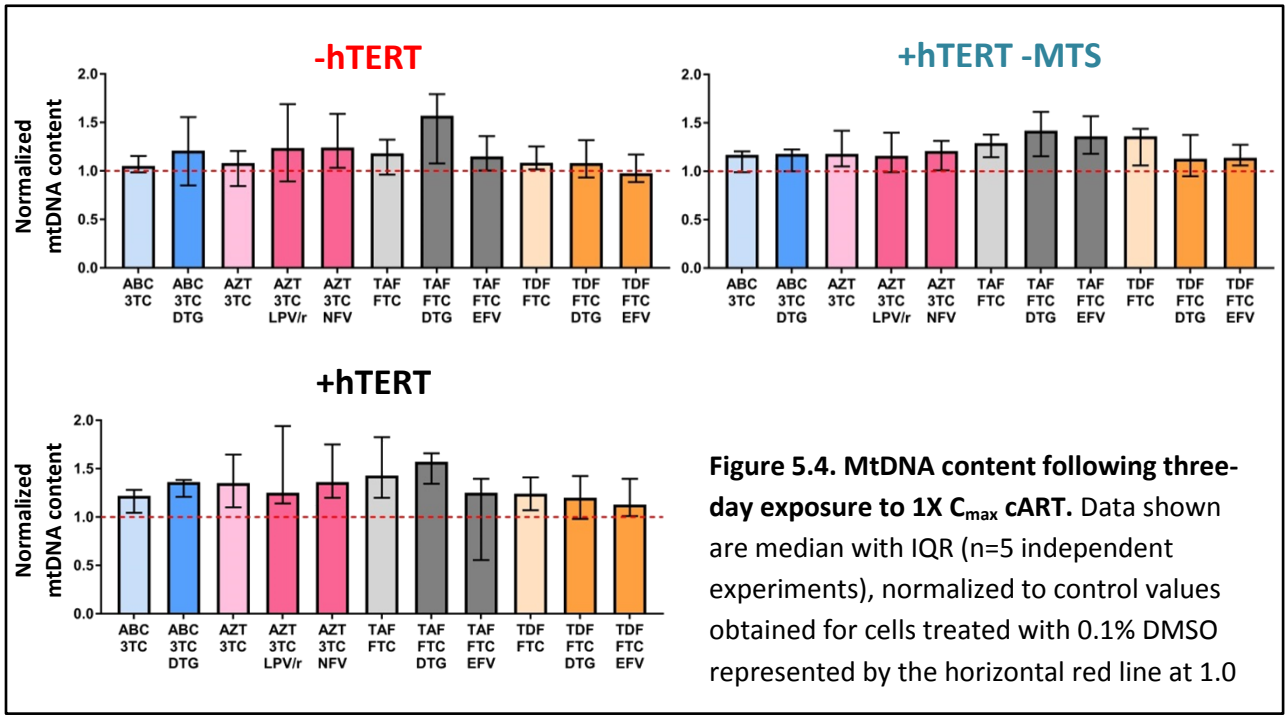

Figure 5.4. MtDNA content following three-day exposure to 1X C_{max} cART. Data shown are median with IQR (n=5 independent experiments), normalized to control values obtained for cells treated with 0.1% DMSO represented by the horizontal red line at 1.0

Taken together, these data seem to suggest that short exposure to cART regimens at 1X C_{max} for three days does not elicit substantial effects on cellular and mitochondrial health. Nevertheless, some of the observed trends, particularly in relation to DTG-exposure, prompted us to examine them more extensively in the longitudinal experiments detailed below.

5.3.2 Longitudinal experiments

5.3.2.1 Cell viability, proliferation and mtDNA content

Data for all time points was obtained from five independent experiments, except for day 15 where n=4, as cells were lost during the culture, due to problems with the CO_2 incubator. A single well of untreated cells (only DMEM medium, no DMSO) for each ALT cell line was maintained throughout the 15-day experiments, and showed that there were no differences between untreated and DMSO-treated cells, for any of the parameters measured. The longitudinal effects of exposure to 1X C_{max} cART regimens on cell viability and proliferation (normalized live cell counts) are shown in Figures 5.5 and 5.6. Cell viability over the treatment and recovery phase was largely unaffected, with the median cell viability across all regimen and all three ALT cell lines remaining above 90% (Figure 5.5). For –hTERT cells exposed to ABC+3TC+LPV/r, the error bars at days 9 and 12 are large, and were primarily driven by a single experiment for which a large proportion of cells were not viable on these days. Overall, these data are still consistent with no overt differences in the viability of cells that were adherent to the culture well.

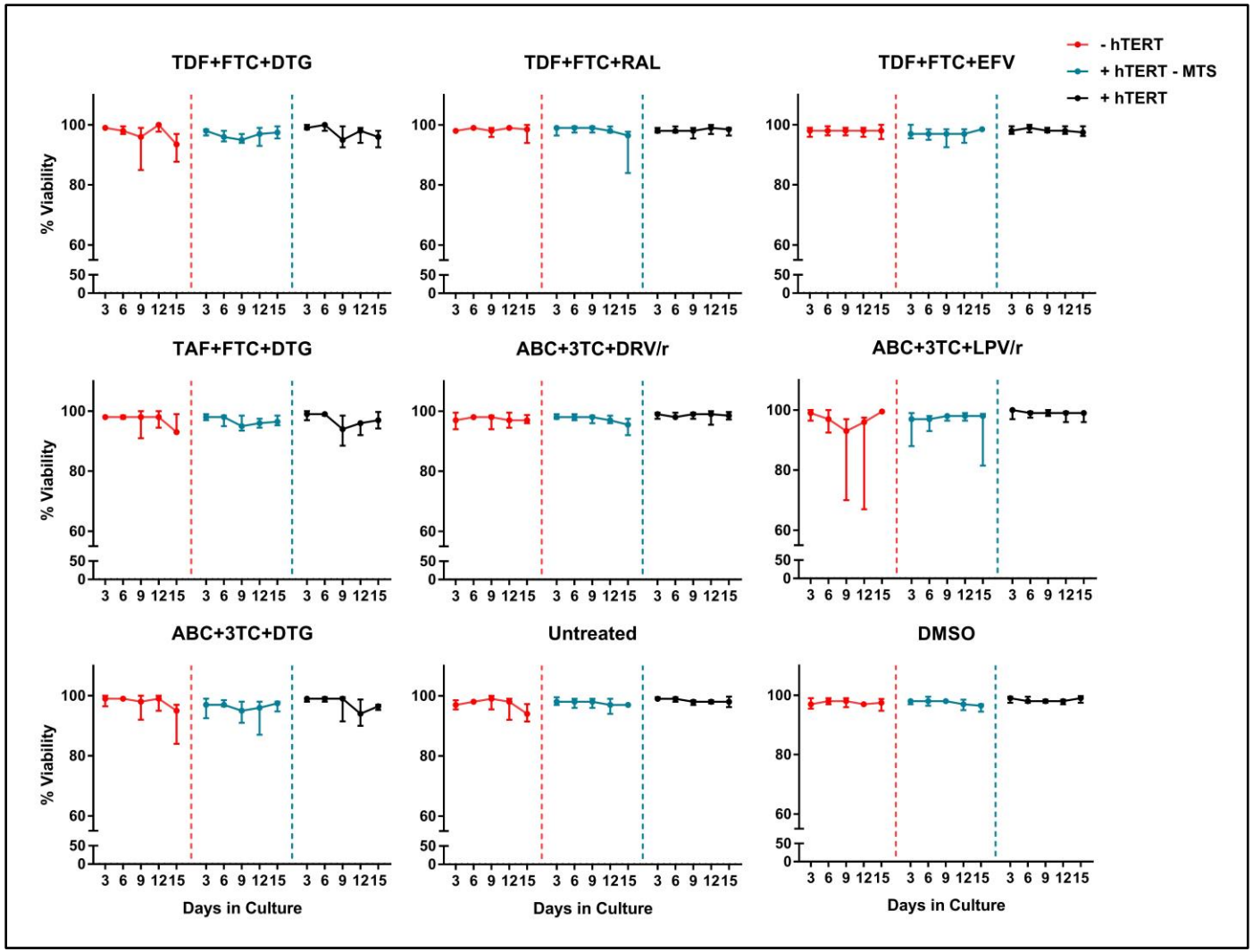

Figure 5.5. Longitudinal changes in cell viability following exposure to 1X Cmax cART regimens for all three ALT cell lines. Cells were cultured in the presence of each treatment for nine days (days 0-9) and then returned to 0.1% DMSO media (except untreated cells) for six days (days 9-15). Data are median with IQR from n=5 independent experiments.

In relation to cell proliferation, it was generally observed that the –hTERT cells grew more slowly than both +hTERT –MTS and +hTERT cells, under any culture condition tested. At day 9, noticeable differences in cell proliferation were observed following exposure to DTG-based regimens (Figure 5.6), as reflected by reduced cell counts. Although limited by sample size, +hTERT and +hTERT -MTS cells exposed to TDF+FTC+DTG, TAF+FTC+DTG and ABC+3TC+DTG each had significantly reduced cell proliferation compared to respective DMSO controls (Mann Whitney $p \leq 0.037$). However, these differences were not statistically significant for –hTERT cells ($p \leq 0.11$), although the trends were similar (Figure 5.6, left panel). Qualitative observations during cell culture ascertained lower confluency and minimal cell death (as indicated by the absence of floating detached cells in culture wells) for cells treated with DTG-containing cART, indicating that the lower cell counts obtained at day 9 were primarily due to reduced proliferation as opposed to increased cell death. The remaining cART regimen did not significantly affect cell proliferation across cell lines.

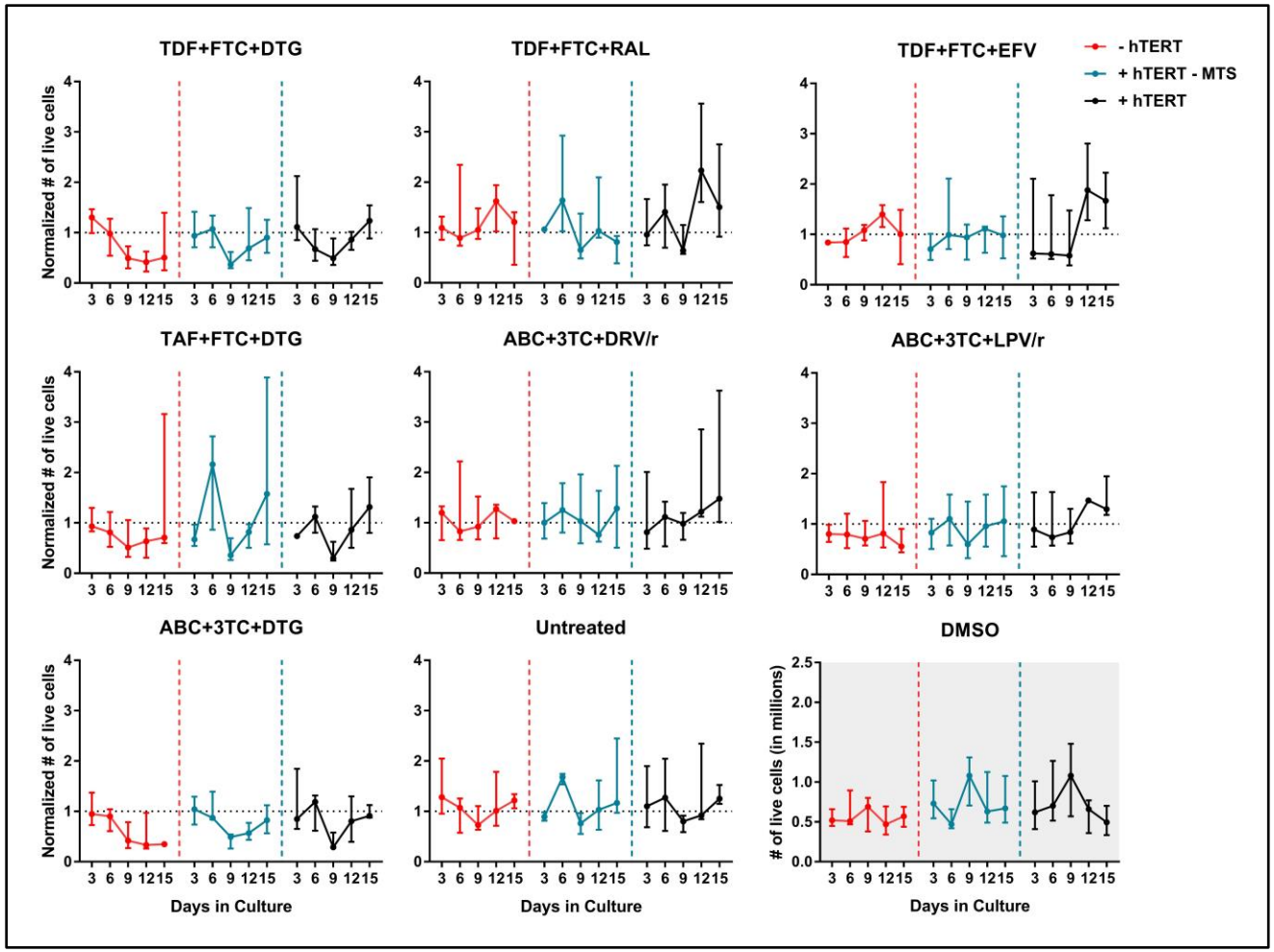

Figure 5.6. Longitudinal changes in cell proliferation following exposure to 1X C_{max} cART regimens for all three ALT cell lines, normalized to corresponding DMSO controls (dashed horizontal lines). Cells were cultured in the presence of each treatment for nine days (days 0-9) and then returned to 0.1% DMSO media (except untreated cells) for six days (days 9-15). Data are median with IQR from n=5 independent experiments. Seeding concentration was 2 x 10^5 cells/well at each time point. Raw data for # of live cells for DMSO controls are shown in the shaded panel (grey).

Table 5.2 shows the average % change in cell proliferation at day 9 (relative to DMSO) for cART regimens across all three ALT cells. The most notable difference were observed for DTG-containing regimens, whereby the mean average decrease across all three DTG-regimens was 55% for –hTERT cells, 61% for +hTERT –MTS cells, and 60% for +hTERT cells. Additionally, while cell proliferation at day 9 was significantly different between TDF+FTC+DTG and TDF+FTC+RAL-exposed –hTERT cells (p=0.02), this difference did not reach significance for +hTERT –MTS (p=0.1) and +hTERT (p=0.2) cells, for which variability appeared greater.

Table 5.2. Average % change in cell proliferation at day 9 (relative to 0.1% DMSO control)

Treatment	- hTERT	+ hTERT -MTS	+ hTERT
TDF+FTC+DTG	**-57**	**-60**	**-49**
TDF+FTC+RAL	+7	-23	-29
TDF+FTC+EFV	0	-40	-34
TAF+FTC+DTG	**-49**	**-61**	**-66**
ABC+3TC+DRV/r	-9	+7	-11
ABC+3TC+LPV/r	-30	-31	-19
ABC+3TC+DTG	**-59**	**-61**	**-65**

*Average % change in cell proliferation for DTG-containing cART is bolded in the table.

Longitudinally, consistent trends were observed whereby cells exposed to DTG-containing regimens showed decreased cell proliferation at day 9 compared to day 3 (Figure 5.6, left panel). Interestingly, following removal of drug pressure, cell proliferation appeared to rebound by day 15 for the +hTERT –MTS and +hTERT cells, and were comparable to cell counts observed at day 3 for the respective treatments. By day 15, cell proliferation was also comparable to the respective DMSO controls. However, for –hTERT cells treated with DTG-containing regimens, cell proliferation appeared to remain decreased on day 15, at the end of the recovery period.

Finally, based on the data collected to date, mtDNA content remained fairly stable during the treatment and recovery phases across all cART regimens, and was comparable to the mtDNA content of DMSO controls at each corresponding time point (Figure 5.7). While there were no overt differences in mtDNA content between ALT cell lines, the data suggests that +hTERT cells exposed to DTG-containing tenofovir regimens may have elevated mtDNA content by day 9 of the treatment phase, which decreased back to control levels by the end of recovery.

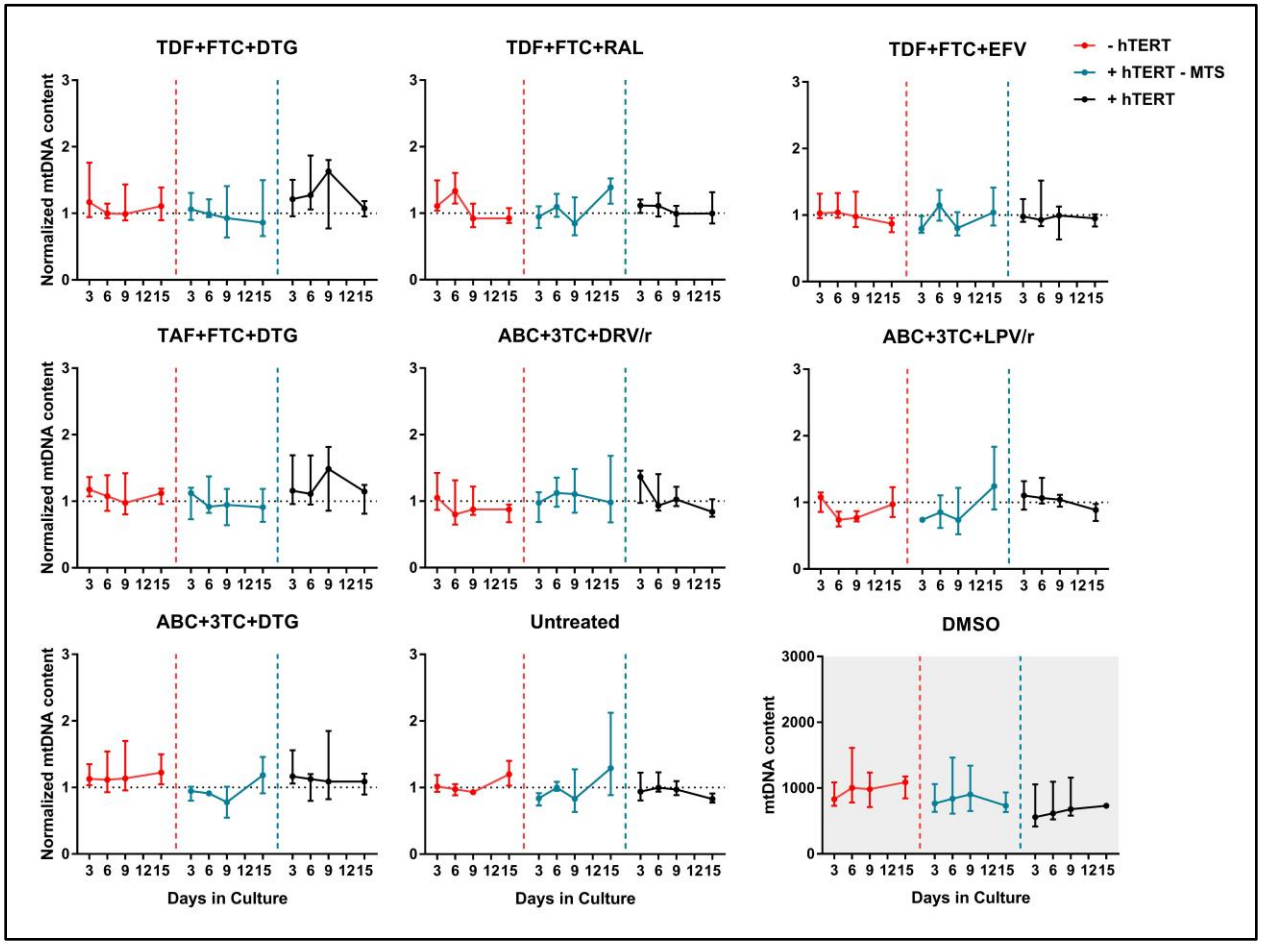

Figure 5.7. Longitudinal changes in mtDNA content following exposure to 1X C_{max} cART regimens for all three ALT cell lines, normalized to corresponding DMSO controls (dashed horizontal lines). Cells were cultured in the presence of each treatment for nine days (days 0-9) and then returned to 0.1% DMSO media (except untreated cells) for six days (days 9-15). Data are median with IQR from n=5 independent experiments. Raw data for mtDNA content of DMSO controls are shown in the panel shaded grey.

5.3.2.2 Apoptosis, mitochondrial mass, MMP and ROS

The analysis of mitochondrial and cellular health was conducted via flow cytometry on day 9 (end of treatment phase) and on day 15 (end of recovery) of the longitudinal experiments. For day 9 flow cytometry, data were available for a total of four independent experiments, as one set of samples was lost (due to human error) during the staining procedure. Day 15 flow cytometry data was available for three of these experiments, as one set of samples was lost during cell culture due to incubator problems.

Results for cell viability and cell proliferation at day 9 for the four flow cytometry sets were unchanged from the full sample (n=5) described previously. Median cell viability was >90%, and cell proliferation was significantly decreased for +hTERT and +hTERT –MTS cells treated with TDF+FTC+DTG, TAF+FTC+DTG or ABC+3TC+DTG compared to the respective DMSO controls ($p \leq 0.029$, data not shown).

All cells were gated for morphology based on light scattering, and total cellular apoptosis was quantified on days 9 and 15 (Figure 5.8). In addition to decreased cell proliferation, all three ALT cells treated with DTG-containing regimens appeared to show increased cellular apoptosis at day 9 compared to cells exposed to non-DTG containing cART. Within cell lines, TDF+FTC+DTG-treatment showed higher total cellular apoptosis than treatment with TDF+FTC+RAL, and these differences approached statistical significance (-hTERT p=0.057, +hTERT –MTS p=0.029, +hTERT p=0.061).

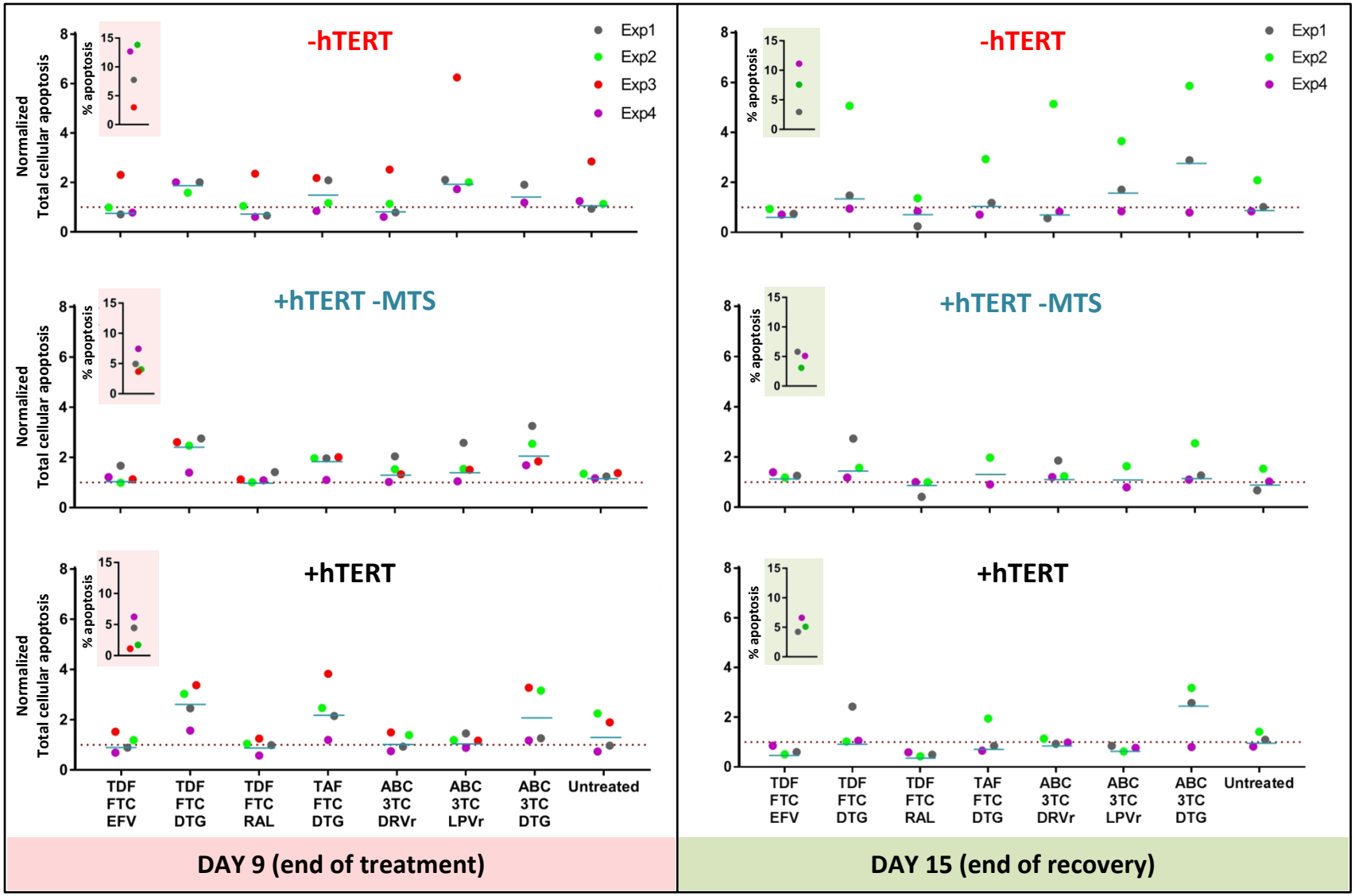

Figure 5.8. Total cellular apoptosis at days 9 and 15, normalized to corresponding DMSO controls (dashed line). Short horizontal blue lines represent the median of each data set. Insets represent raw % total cellular apoptosis (Annexin V staining) for the DMSO controls of each experiment.

Similar results were obtained for cellular apoptosis levels quantified within live cell populations that were determined based on viability gating (Figure 5.9). Additionally, for a given cART regimen, the levels of apoptosis (both total and among live cells) were not significantly different between the three cell lines. The only exception to this were cells treated with ABC+3TC+LPV/r, whereby –hTERT cells had significantly higher levels of cellular apoptosis compared to +hTERT cells ($p=0.03$), but not +hTERT –MTS treated cells ($p=0.20$). This may suggest a potential protection by hTERT that is capable of translocating to the mitochondria. Lastly, any observed increase in cellular apoptosis during the treatment phase appeared to resolve during the recovery phase (by day 15) across all cell lines (Figures 5.8 and 5.9).

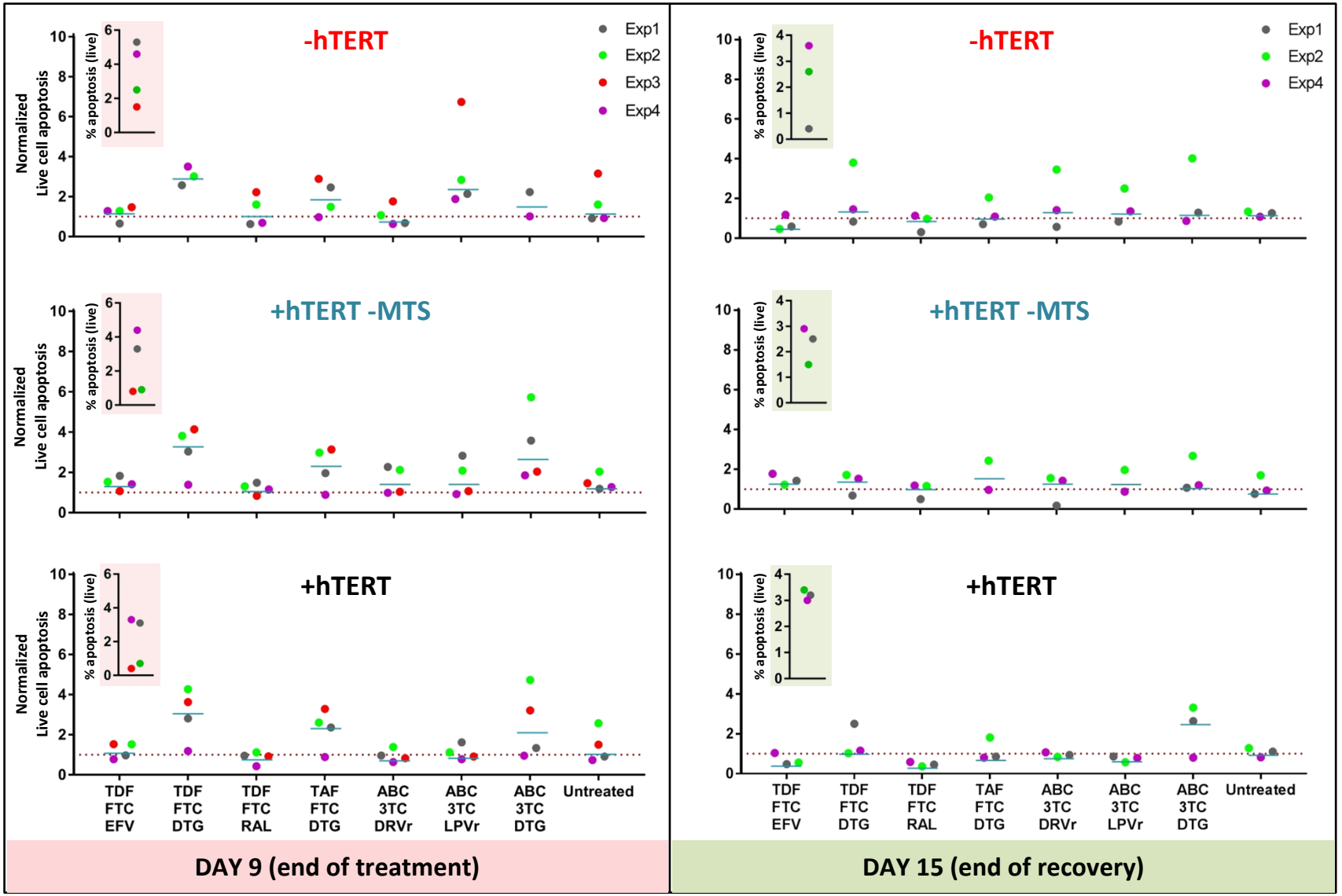

Figure 5.9. Cellular apoptosis among live cells at days 9 and 15, normalized to corresponding DMSO controls (dashed line). Short horizontal blue lines represent the median of each data set. Insets represent raw % live cell apoptosis (Annexin V staining) for the DMSO controls of each experiment.

Interestingly, the increased apoptosis observed with exposure to DTG-containing regimen was accompanied by an increase in mitochondrial ROS in all three ALT cells (Figures 5.10 and 5.11A-B). Compared to DMSO controls, differences reached statistical significance for +hTERT –MTS cells ($p \leq 0.03$), but the effects were also noteworthy for +hTERT ($p=0.061$) and –hTERT cells ($p=0.19$).

There was no obvious evidence for hTERT-mediated protection against DTG-associated ROS. Compared to TDF+FTC+RAL, treatment with TDF+FTC+DTG induced significantly higher ROS in both +hTERT –MTS ($p=0.029$) and +hTERT ($p=0.06$) cells. Subtle differences were observed between ALT cell lines in relation to ABC+3TC+LPV/r-induced ROS, whereby –hTERT and +hTERT –MTS cells had a median 71% and 69% increase in ROS respectively, while for +hTERT cells the median increase was 21%. Likewise, for DRV/r regimen, the median increase in ROS was 34%, 37% and 17% for –hTERT, +hTERT –MTS and +hTERT cells respectively. These may suggest a potential protection against PI/r-induced ROS by mitochondrial telomerase. Lastly, any increase in ROS observed with cART treatment appeared to resolve by the end of recovery (day 15) for all three cell lines; the only exception being –hTERT cells treated with PI/r-containing regimen that continued to show marginally elevated ROS even during the recovery phase.

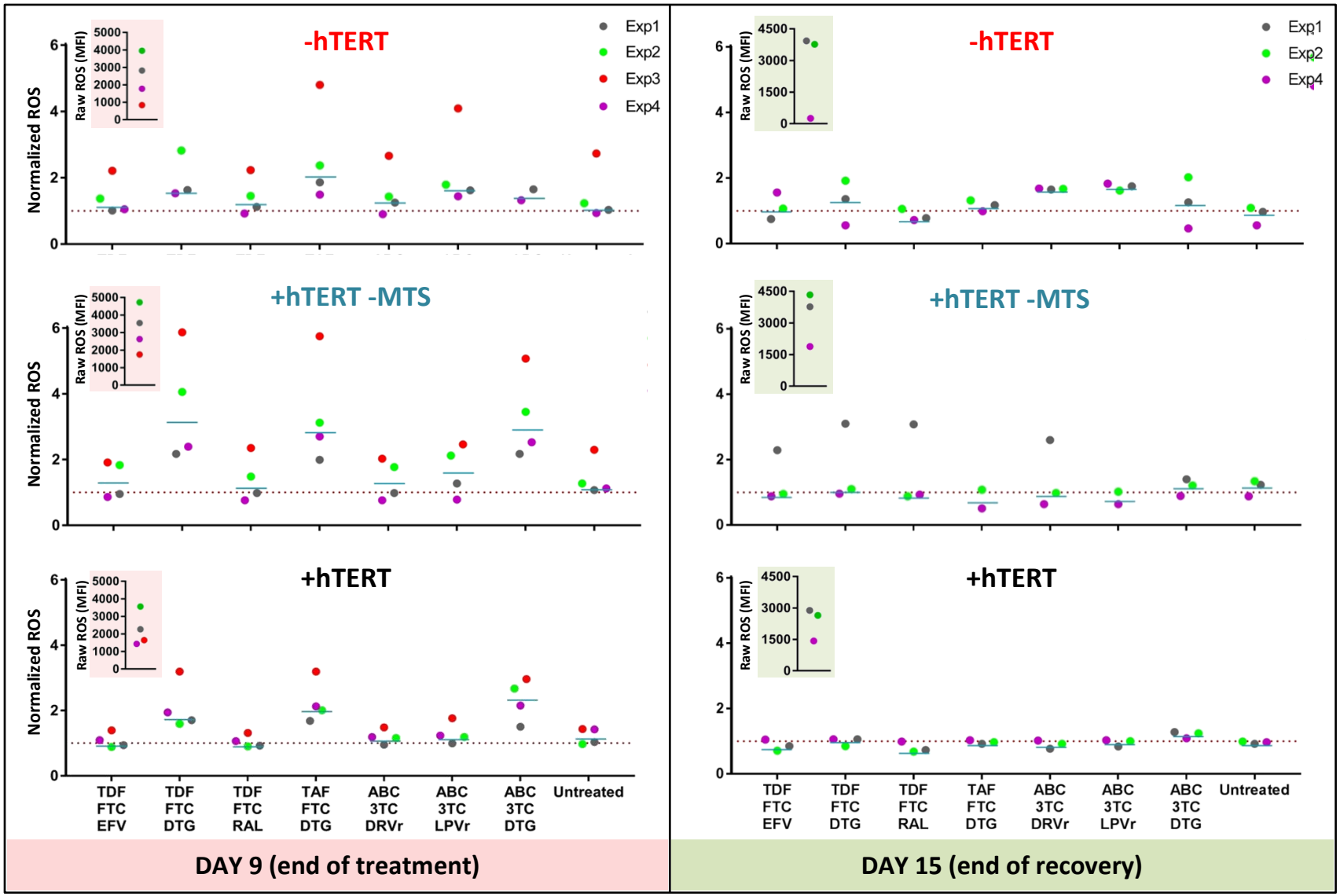

Figure 5.10. Mitochondrial ROS at days 9 and 15, normalized to corresponding DMSO controls (dashed line). Short horizontal blue lines represent the median of each data set. Insets represent raw MFI values for MitoSOX Red (ROS), for the DMSO controls of each experiment.

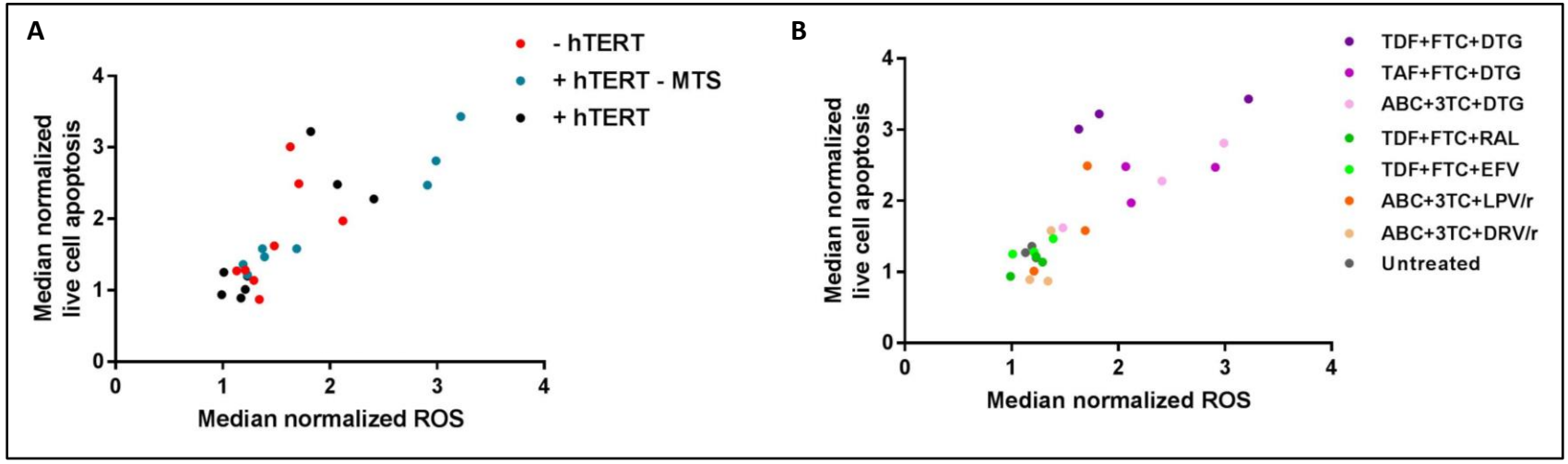

Figure 5.11. Correlation between median normalized cellular apoptosis and median mitochondrial ROS among all live cells. Data shown is the overall correlation of the median values obtained from n=4 independent experiments for each condition, with data separated by (A) ALT cell line, and (B) type of cART regimen. Spearman's rho=0.82, $p<0.0001$.

Lastly, mitochondrial mass and mitochondrial membrane potential (MMP) at day 9 were relatively similar across regimens, and across cell lines (Figures 5.12 and 5.13). In accordance with effects on apoptosis and ROS, ALT cells treated with DTG-containing regimens appeared to have slightly higher mass and MMP compared to DMSO controls. As before, these effects were reversible following removal of drug pressure. Significant positive correlations between mitochondrial mass and MMP were observed for all three ALT cell lines ($p<0.001$, Figure 5.14).

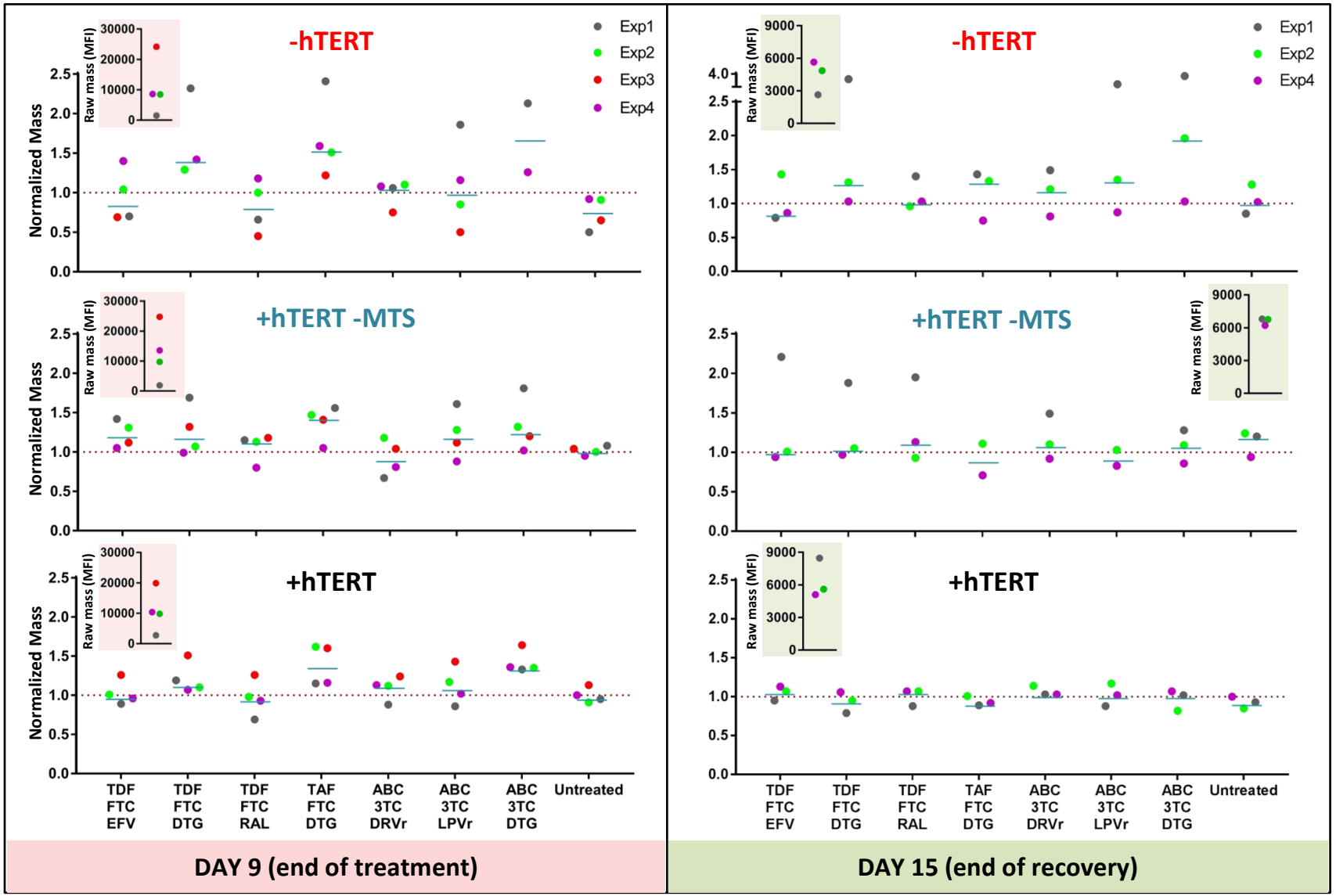

Figure 5.12. Mitochondrial mass at days 9 and 15, normalized to corresponding DMSO controls (dashed line). Short horizontal blue lines represent the median of each data set. Insets represent raw MFI values for MitoTracker Green FM (mitochondrial mass), for DMSO controls of each experiment.

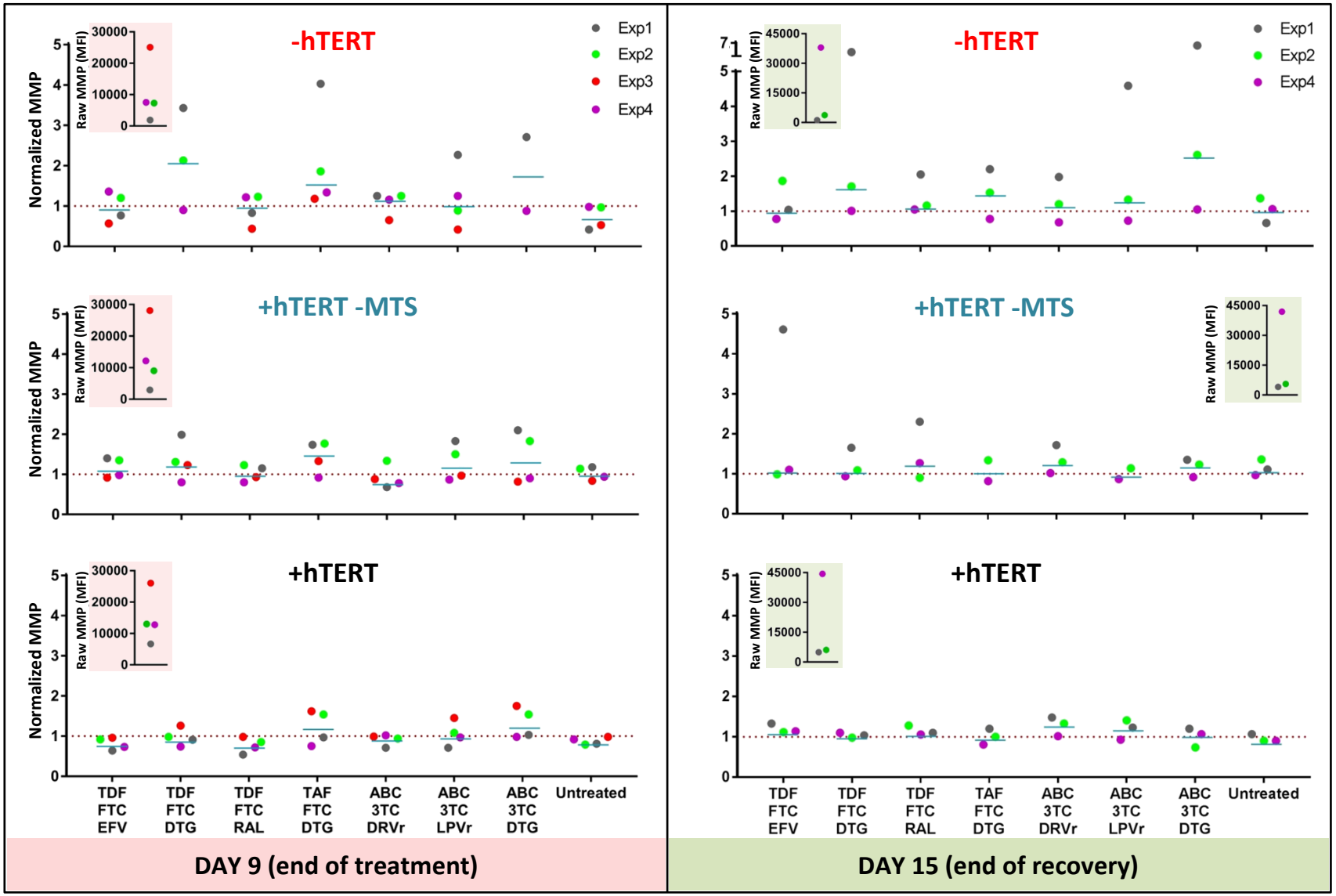

Figure 5.13. MMP at days 9 and 15, normalized to corresponding DMSO controls (dashed line). Short horizontal blue lines represent the median of each data set. Insets represent raw MFI values for MitoTracker Deep Red FM (MMP), for the DMSO controls of each experiment.

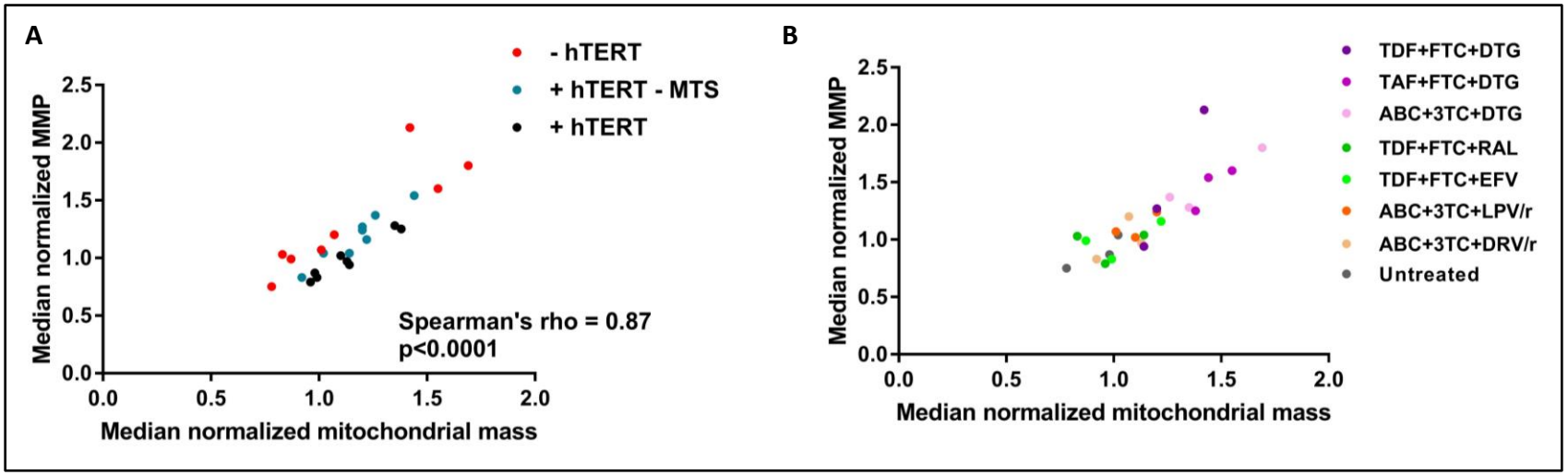

Figure 5.14. Correlation between median normalized mass and median mitochondrial MMP among all live cells. Data shown is overall correlation of median values obtained from n=4 independent experiments, with data separated by (A) ALT cell line, and (B) type of cART regimen. Spearman's rho=0.86, p<0.0001.

5.4 Discussion

In the context of lifelong cART for the treatment of HIV, it is extremely important to evaluate the long-term safety of current and new classes of ARVs, both at the organismal and cellular levels. Despite considerable knowledge gained from clinical studies of HIV-infected cART-treated individuals, trials usually contain no placebo groups or HIV-uninfected groups, and hence it becomes challenging to differentiate between the effects of HIV infection *vs.* those of cART. Given the incorporation of newer InSTIs into current first-line therapy, there is an urgent need for data on drug toxicities, to ensure safety for all, including in pregnancy and neonatal development. In view of this, I designed *in vitro* experiments to evaluate the cellular and mitochondrial toxicities of seven cART regimens important in the context of pregnancy, and determine whether hTERT protects against any cART-associated mitochondrial toxicities.

In this study, I demonstrate that nine-day DTG treatment in combination with dual NRTI backbones at pharmacologically relevant concentrations is associated with reduced cellular proliferation, without any noticeable cytotoxic cell death during culture. Further, among live cells obtained at the end of treatment, we observe increased levels of cellular apoptosis in conjunction with increased levels of mitochondrial ROS. Taken together, these are indicative of increased cellular and mitochondrial toxicity associated with DTG-based treatment. Although PIs are known to produce ROS, and increase oxidative and endoplasmic reticulum (ER) stresses in cell culture models [138,324,332], this is the first report of increased ROS in association with the use of an InSTI drug, to our knowledge. While the mechanism for this remains unclear, it is noteworthy that the observed association was specific to DTG-containing regimens and was not observed with RAL, when comparing the two ARVs on the same backbone of TDF+FTC. This is

in agreement with another study reporting an absence of endoplasmic reticulum (ER) stress and inflammatory responses in macrophages following treatment with RAL. On the contrary, RAL was shown to reduce PI-mediated oxidative and ER stresses [332]. Together with my data, this may suggests that RAL is safer to overall cellular health, compared to DTG.

Furthermore, the presence of hTERT capable of translocating to the mitochondria did not appear to reduce DTG-associated ROS in these cells. Studies have shown that the most common stimulus for mitochondrial transport of hTERT is oxidative stress, either extrinsic, caused by H_2O_2 treatment, hyperoxia or irradiation [159,333,334] or intrinsic, such as OXPHOS-associated increase in cellular ROS levels [158,335]. More importantly, these studies also demonstrated that mitochondrial localization of hTERT resulted in decreased cellular oxidative stress, lower nuclear and mitochondrial DNA damage, and lesser apoptosis in most cell types, including fibroblasts and endothelial cells [158,159,163,333–336]. In agreement with these findings, we too observed that mitochondrial hTERT appears to reduce PI-mediated ROS and cellular apoptosis. However, it remains unclear why the same protection was not conferred against DTG-mediated cellular stresses, suggesting that there could be other mechanisms involved with the latter.

Along with increases in apoptosis and ROS, treatment with DTG-containing cART was shown to increase mitochondrial mass and MMP. A few studies have reported increased mitochondrial mass, possibly a result of increased mitochondrial biogenesis, in response to stresses related to ROS and irradiation [337,338]. Treatment with older NRTIs such as d4T, ddI and AZT have been shown to reduce mitochondrial mass and deplete mtDNA content in HIV-

infected individuals and in cultured human cells [77,103,111,115,117,119,121]. In our study, newer NRTIs such as ABC, TDF, and FTC did not exert mitochondrial and cellular toxicities, a finding that is consistent with other *in vitro* studies reporting on the toxicities of these drugs at clinically relevant concentrations [77,102,133]. These results add further credence to the comparative safety of newer NRTIs. Additionally, we did not observe any noticeable changes in fibroblasts' mitochondrial mass or mtDNA content following EFV treatment, which is in contrast with a previous study that reported increased mitochondrial mass, altered mitochondrial morphology and apoptosis in cultured hepatocytes following treatment at low to moderate concentrations of EFV [155]. Our lab also recently observed increased mtDNA content and changes in mitochondrial morphology following EFV treatment in lymphoblast cells, suggestive of upregulated mitochondrial biogenesis [328]. Together, this suggests that the effects of EFV may be cell-specific. Interestingly, we observed that exposure to DTG-containing regimens in +hTERT cells appeared to increase mtDNA content in addition to increasing mitochondrial mass, providing support to the theory of stress-related compensatory organelle biogenesis.

Although mitochondrial hTERT appeared to protect only against PI-mediated stresses in this study, qualitative and quantitative observations from cell culture revealed that the presence of telomerase components in both +hTERT –MTS and +hTERT cells appeared to confer a growth benefit, compared to the –hTERT cells. This agrees with a previous study on these cells that reported telomerase-mediated acceleration of cell growth kinetics [331].

Lastly, through the longitudinal design of the study, it was determined that cART-associated cellular and mitochondrial effects were mostly reversible following the removal of drug pressure. This reinforces the notion that the observed changes in cell proliferation, apoptosis, ROS, mitochondrial mass and MMP were the result of treatment-induced stress, and that tissues can adapt when stressors are removed. Although mtDNA content remained fairly stable during the treatment and recovery phases, it is possible that the increased ROS associated with certain treatments may have resulted in the accumulation of mutated mtDNA. We observed a rebound in cell proliferation following removal of drug pressure, which could potentially promote clonal expansion of pre-existing mutations without considerably altering mtDNA levels. This phenomenon merits further investigations related to the quantification of somatic and heteroplasmic mtDNA mutations following cART exposure, to help explain the 'mutation theory of mitochondrial aging' as suggested previously [96].

5.5 Strengths and Limitations

The strengths of this study extend beyond the longitudinal design, which enabled a better understanding of changes in mitochondrial and cellular dynamics following treatment and removal of cART pressure. My study is the first to address potential toxicities associated with DTG treatment, and also evaluated cART regimens relevant to pregnancy. This is extremely important, as current guidelines are shifting towards InSTI-based regimens, and women are treated with lifetime cART compared to historical practices, where women often initiated cART in the second trimester and may have stopped therapy post-delivery. However, my study has its limitations. It was conducted in transformed cell lines that were made to express telomerase components. Although cART-associated toxicities in patients usually manifest in metabolically

active tissues such as brain, muscle, liver, pancreas and adipocytes that are highly dependent on mitochondrial function, it is likely that the cell lines used in this study have different cellular bioenergetics compared to primary cells or stem cells that naturally express telomerase. Therefore, the data obtained might not be directly translatable to humans. This calls for similar investigations of cART toxicities in other cell types, including human stem cells and other primary cells. The recent evidence of increased neural-tube defects associated with DTG exposure during pregnancy adds to the importance of such investigations, given that neural-tube development from stem cells happens early during fetal development, a time when progenitor cells are most vulnerable to toxicities. Another limitation is the amount of variability in mitochondrial metrics quantified, arising from both intrinsic (cell culture) and extrinsic (flow cytometry) sources. A larger number of independent replicates would be desirable to combat this, something that is planned in the coming months.

5.6 Conclusions

In conclusion, I observed that treatment with DTG-containing regimens results in apparent cellular and mitochondrial toxicities, as reflected by decreased cell proliferation, as well as increased mitochondrial mass, MMP, ROS and cellular apoptosis. Further, these effects appear to resolve following removal of cART pressure, suggesting that they may be at least partially reversible. Additionally, my study suggests that RAL could be a safer option for InSTI-based therapy, given the lack of any toxicities observed with its use. Given that DTG is currently more widely used than RAL globally, these results merit further investigations using a variety of cell types, as well as animal models, to evaluate the cytotoxicity of clinically relevant ARVs.

Chapter 6: CONCLUSIONS

6.1 Summary of Findings

My research primarily sought to address the effects of cART exposure on biomarkers of cellular aging, in both clinical specimens and cell culture models. The first objective was to measure LTL and blood mtDNA content at birth, and over the first three years of life, among *in utero* cART-exposed HEU children and HUU control children. The second objective was to investigate alterations in mitochondrial mass, MMP, ROS, cellular apoptosis and mtDNA content, reflective of mitochondrial and cellular health, following exposure to (and recovery from) cART regimens that are currently extensively used in pregnancy, both in North America and throughout the world.

I found that HEU LTL at birth, and over the first three years of life, was similar to HUU LTL, and was not negatively affected by *in utero* exposure to maternal cART and/or postnatal AZT prophylaxis. These are indeed reassuring findings. Instead, maternal smoking during pregnancy appeared to affect infant LTL at birth. I detected a significant interaction between HEU/HUU status and maternal smoking during pregnancy, whereby the latter was associated with shorter LTL in HEUs but longer LTL in HUUs. While the effect of smoking on HEU LTL may be related to oxidative stress, in addition to HIV/cART-related stresses, the counterintuitive observation among HUUs may be explained by possible turnover of leukocytes with shorter TL, resulting in residual cells with an apparent longer LTL phenotype. This observation warrants further investigations on the effects of tobacco exposure during pregnancy on TL. Lastly, longitudinal assessment of HEU LTL demonstrated that LTL rapidly declined during the first

year of life, and slowed thereafter, possibly reflecting a period of significant growth, as well as differentiation and maturation of immune cells.

Given the observed association between smoking during pregnancy and longer HUU LTL at birth, I conducted similar investigations in other HUU infant tissues, including umbilical cord blood and cord tissue, placenta and mouth epithelial cells. I observed consistent univariate associations between smoking and longer TL in all tissues investigated, and some of these effects persisted after adjusting for other covariates. These findings may suggest that the effect of smoking is systemic and not restricted to blood cells, and stresses the importance of smoking cessation interventions, especially during pregnancy and among women of reproductive age.

Unlike LTL, HEU mtDNA content at birth was higher than that of HUU children, and remained elevated up to age three. Further, type of cART was found to be associated with mtDNA content at birth, with HEUs exposed *in utero* to PI/r-based cART having higher mtDNA content than HUU children. The HEU/HUU status*maternal smoking interaction term suggests that smoking is associated with higher mtDNA content at birth only among HUU children. Longitudinally, HEU mtDNA content was relatively unchanged during the first six months of life and gradually declined thereafter.

Taken together, my findings indicate that only mtDNA content, and not LTL, is affected by *in utero* exposure to HIV/cART among HEU children. Other exposures, such as exposure to maternal smoking during pregnancy, appear to affect both LTL and mtDNA content. Consistent with national estimates, we observed a ~2-fold risk of being born preterm among HEU children,

compared to HUU children. In addition, we observed an increased risk of preterm birth in association with maternal smoking during pregnancy. In this cohort, preterm birth was further associated with longer LTL and higher mtDNA content at birth. Although other studies report an association between the use of PI-based cART and increased risk of preterm birth, I did not detect any such relationship in this cohort. Nevertheless, the relationship between preterm birth, type of cART regimen, longer LTL, and higher mtDNA content requires further investigations, particularly to tease apart the effects of smoking exposure *vs.* being born preterm.

Although much knowledge has been gained from these two clinical investigations of aging markers in HEU children, it is often challenging to distinguish between the effects of *in utero* exposure to maternal HIV milieu *vs.* exposure to cART. Given that certain cART regimens were shown to affect mtDNA content in this population, there is an urgent need to determine the long-term safety of both currently used and newer classes of ARVs. To address this knowledge gap, I designed *in vitro* experiments to evaluate the cellular and mitochondrial toxicities of current and clinically relevant cART regimens at pharmacological concentrations. Further, I aimed to investigate the potential protective role of hTERT in modulating mitochondrial toxicities (if any), using a telomerase-independent cell line.

I showed that DTG-containing cART regimens decreased cell proliferation without causing substantial cell death. Further, treatment with DTG-containing cART was associated with increased mitochondrial mass, MMP, ROS and cellular apoptosis. Together, these are reflective of DTG-associated mitochondrial and cellular toxicity, and my study is the first to demonstrate this. Of note, I did not detect any toxicity with RAL-based regimens, suggesting that

certain InSTIs may be safer than others. Consistent with other studies, PI/r-based regimens were associated with increased mitochondrial ROS. Interestingly, the presence of mitochondrial hTERT appeared to reduce PI- but not DTG-mediated ROS. This suggests that there could be other mechanisms associated with the latter. Treatment with DTG-containing cART also appeared to increase mtDNA content in some instances which, along with the observed increase in mitochondrial mass, may reflect compensatory organelle biogenesis and mtDNA replication in response to cellular stress and/or damage. This, in turn, could promote the clonal expansion of mtDNA mutations, and could result in further mitochondrial dysfunction and/or exacerbated mitochondrial health. This clearly warrants further investigations. Finally, the longitudinal design of my study helped establish that cART-associated toxicities were mostly reversible upon removal of drug pressure. In summary, my study suggests that some currently used cART regimens can affect cellular and mitochondrial health, and that hTERT may be protective against some forms of toxicity. This highlights the need to investigate the effects of newer cART regimens in other cell types, for example, cells that naturally express telomerase (hTERT) such as placenta and stem cells.

6.2 Significance and Translation of the Study

Several pathologies related to mitochondrial dysfunction/toxicity, premature aging and cellular senescence have been reported in HEU children. LTL and mtDNA content are two easily quantifiable biomarkers of cellular aging, and have been linked to several of these pathologies in children and adults LWH.

My study provides detailed insights into the dynamics of LTL and mtDNA content during the early life of HEU children, demonstrating that cART and smoking affect these two biomarkers to varying extents. Further, my study is the first to report on the longitudinal changes in HEU LTL and mtDNA content in early life, and presents an overall picture of these biomarkers in this population. The observations regarding the effects of smoking adds to the current knowledge on the negative impact of prenatal exposure to tobacco, emphasizing the benefits of smoking cessation. Although in our cohort LTL is unaffected by *in utero* cART exposure, a reassuring finding, a South African study recently reported shorter LTL in cART-exposed HEUs compared to HUUs [221]. My study further showed that certain regimens affect mtDNA content more than others. This is corroborated by my *in vitro* experiments, whereby newer cART regimens that are now extensively used in pregnancy also affected mitochondrial and cellular health.

Taken together, these observations could be relevant to future epidemiological studies investigating the predictive value of infant TL and mtDNA content on health outcomes in this ever-expanding population. My findings could also inform future clinical trials and treatment guidelines pertaining to the treatment of HIV.

6.3 Future Direction

Through my research, I showed that ARVs affect mitochondrial health and mtDNA content. Regarding the latter, aside from quantity, mtDNA quality could also affect mitochondrial function. A logical next step would be to ascertain mtDNA somatic and heteroplasmic mutations following cART exposure, using the cell culture samples already obtained. As mentioned before, potential compensatory mitochondrial biogenesis in relation to cART-stresses would provide the perfect platform for clonal expansion of mtDNA somatic mutations. Our lab has developed an ultra-deep sequencing assay to quantify these mutations, and these will be conducted in the near future.

Our cohort study regarding LTL and blood mtDNA content dynamics during the early life of HEU and HUU children has certain limitations. The imbalance in ethnicity is a potential confounder as some ethnic groups have been reported to have longer LTL. Furthermore, the lack of data on smoking frequency, duration and intensity affects our ability to fully estimate the effect of maternal smoking during pregnancy on these two biomarkers of aging, and the role that timing and intensity may play. Future studies should replicate these findings in a more balanced cohort, with exhaustive data on smoking habits as well as other substance use during pregnancy such as alcohol, cannabis, and other drugs. The aforementioned concerns could be reduced if the study took place in an African cohort of HEU and HUU children, where women are less likely to use substances, and where ethnicity would be much more homogeneous. Further, given our observations on LTL and mtDNA in early life, it would be important to follow young HEU children longitudinally into adolescence and early adulthood, to determine whether these effects persist and/or predict health outcomes later in life. Alternatively, if following these same

children is not possible, future studies could also investigate these two biomarkers among older HEU and HUU children.

HIV treatment guidelines are constantly evolving, and PLWH, including pregnant women and children are now exposed to newer classes of HIV ARVs. Specifically, InSTIs are increasingly incorporated into current first-line regimens and there is a dearth of data regarding their long-term safety and toxicities. Given our preliminary *in vitro* results on the apparent mitochondrial toxicities of DTG exposure, future clinical studies should also include a cohort of HEU children exposed to InSTI-containing cART during pregnancy and/or HIV+ adolescents receiving InSTI-cART, to increase knowledge about the safety of these drugs.

Additionally, the observed *in vitro* effects of cART, together with the protective effects of hTERT, warrant replication in cell types that naturally express telomerase. My initial proposal was to conduct similar investigations in a human embryonic stem cell line, as effects observed in such a cell line would relate to potential effects of conceiving while on cART. Given that current treatment guidelines recommend the initiation of lifelong cART at HIV diagnosis, most women will now conceive while already on therapy, and hence most HEUs will have been exposed throughout gestation. Therefore, such studies would be of critical importance to further evaluate the long-term safety of cART regimens used in pregnancy on the health of HEUs. Another graduate student in our lab is currently adapting my experimental protocol, including the combination of cART regimens to be tested, in a human embryonic stem cell line.

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