PLACENTAL MITOCHONDRIAL DYSFUNCTION IN RELATION TO PRETERM DELIVERY IN HIV PREGNANCY

by

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Abstract

Background: Preterm birth (PTB) (<37 weeks of gestation), is the leading cause of mortality and morbidity among children, responsible for >1 million deaths in 2015 [1]. In North America, PTB occurs in 6–10% of births. However, among pregnant women living with HIV, the rates are higher (18-29%). To date, there is no generally accepted mechanism underlying such increased rates.

One possible explanation is reduced maternal progesterone production during pregnancy, which may be related to HIV infection and/or antiretroviral (ARV) treatment. Synthesis of progesterone (hormone central to pregnancy maintenance), is dependent on placental mitochondrial function. Given that many ARVs can affect mitochondrial (mt) function**, I investigated** the possible effects of ARV on placental mtDNA content and progesterone levels.

Methods: 136 HIV+ and 60 HIV- pregnant women were enrolled in the Canadian prospective study, the **C**hildren and women: **A**nti**r**etroviral and **M**arkers of **A**ging (**CARMA**) cohort. Placenta and blood specimens, as well as clinical and sociodemographic data, were collected. Placental and plasma progesterone levels, as well as placenta mtDNA content, were measured using ELISA and qPCR respectively. We extended these investigations of ARV effects to *in vitro* models on two human placental cell lines, JEG-3 and BeWo.

Results: Within this cohort, HIV-exposed uninfected (HEU) infants were born at an earlier gestational age (p=0.017), with a lower birth weight (p=0.011) compared to controls. PTB showed no association with HIV status, placenta mtDNA or progesterone levels. However, higher mtDNA was associated with preeclampsia (p<0.001), which often leads to PTB.

Furthermore, placenta mtDNA and progesterone levels were significantly negatively correlated to one another (n=196, rho=-0,242, p<0.001). With respect to ARV exposure during pregnancy, having received a nonboosted-PI PI (23% vs. boosted-PI (66%)(p=0.008), and having had a cesarian section (vs, vaginal delivery, p=0.037) were associated with higher placenta mtDNA.

Conclusion: My results suggest that placental mtDNA content may become elevated as a possible compensatory mechanism for placental dysfunction in women with pregnancy complications.

Lay Abstract

Being born too early (preterm) accounted for approximately 1 million infant deaths in 2015. While 9% of births in North America are preterm, this rises to (18-29%) among pregnant women living with HIV. The cause for this is yet unknown.

One reason might be the anti-HIV drugs. Although they prevent the child from getting infected with HIV, some seem to damage the DNA in the mitochondria (cellular organelles in charge of a cell's energy production). If the DNA is damaged, the cell will not work properly. Anti-HIV drugs also affect the mother's ability to make progesterone, a hormone needed during pregnancy. If the amount of progesterone is insufficient, the risk of preterm birth increases.

We studied whether HIV+ women have some damage in their placenta DNA than HIV- women do. We also examined if any HIV drugs were related to unusual levels of progesterone and DNA in the mitochondria.

Preface

The contents of this thesis are my own original work, including all the images and figures here presented. All the experiments were designed by me in conjunction with my supervisor, Dr. Hélène Côte. The University of British Columbia Research Ethics Board (H08-02018) approved all conducted research. Written consent was received from all enrolled participants. A copy of the consent form is attached in the appendix section. A large number of research staff at Oak Tree Clinic performed data collection. Beheroze Sattha assisted in data organizing and data entry into the database.

Izabelle Gadawski, a lab technician in Dr Cote's lab, carried out tissue handling and extraction. Some ELISA and RT-PCR runs were performed by Colin Orth, a volunteer undergraduate student that worked under my supervision.

I conducted most of the measurements described in Chapters 2 and 3, organized the data, and performed all the statistical analyses presented in this dissertation.

A version of this study is currently being prepared in manuscript format to be published after the completion of my graduate program.

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List of Abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immune Deficiency Syndrome
ALB	Albumin
ATP	Adenosine Triphosphate
AZT	Zidovudine
BMI	Body Mass Index
BC	British Columbia
bp	Base pairs
CARMA	Children & Women: AntiRetrovirals and Markers of Aging
CART	Combination Antiretroviral Therapy
CCR5	C- Chemokine Receptor 5
CD4	Cluster of Differentiation 4
C_{max}	Maximum Concentration
CV	Coefficient of Variation
C/S	Caesarean Section
DMSO	Dimethyl Sulfoxyde
DNA	Deoxyribonucleic Acid
DRV	Darunavir
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid

EFV	Efavirenz
ETR	Etravirine
FDA	Food and Drug Administration
FTC	Emtricitabine
GDM	Gestational Diabetes Mellitus
GIH	Gestational Induced Hypertension
HIV	Human Immunodeficiency Virus
IC	Internal Control
IDV	Indinavir
IQR	Interquartile Range
IUGR	Intra Uterine Growth Restriction
LPVr	Ritonavir boosted-Lopinavir
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTCT	Mother-To-Child-Transmission
nDNA	Nuclear DNA
NFV	Nelfinavir
NNRTI	Non-Nuclease Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
P4	Progesterone
P5	Pregnenolone
PE	Preeclampsia

PTB	Preterm Birth
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
pVL	Plasma Viral Load
QC	Quebec
qPCR	Quantitative Polymerase Chain Reaction
RAL	Raltegravir
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RTV	Ritonavir
SD	Standard Deviation
SGA	Small for Gestational Age
TDF	Tenofovir Disoproxil Fumarate
TGF-β	Transforming Growth Factor-beta
tRNA	Transfer-RNA
UNAIDS	Joint United Nations HIV/AIDS program
WHO	World Health Organization

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Firstly, I would like to thank my supervisor, Dr. **Helene Cote** for all her guidance, feedback, support, encouragement, patience and sincere care, all throughout this project. Her mentorship was essential for my development as a student and as a professional scientist. She polished my edges and taught me to think as a real scientist, because "she is not here to spoon-feed me". Her honesty and frankness were always much appreciated and well-received. Helene, I admire you and I will always remember you.

Secondly, I want to especially thank my lab partners and really good friends **Abhinav**, **Anthony,** and **Sara** for their support, their patience, and their endless and tireless help. A big Thank you guys for have been always there for me. I could not quantify how much I have learned from the both of you. Working with you for these last two years have been a sincere pleasure. You make the lab, a positive place to work in. You will be missed.

Also, my beloved **Izabelle**, she is the heart and soul of the lab. Without her, our lab would fall apart. Thank you for caring, for listening to me, for your patience and help. You cheer up the lab. Also, I want to especially thank **Beheroze**, she has been there for me, promptly and ready to help anytime I needed it. Also thank you, **Colin**, for having volunteered on my project, I hope I got to inspire you with my love for research. Thanks also to **Moh**, my lover, life partner, and best friend. Thanks for your moral support. I hope to have you next to me sharing many more triumphs to come.

Overall, to all of you, many thanks for the countless hours you spent training, teaching, and troubleshooting with me. I would not have been able to succeed without you. I definitely could not have asked for a better group of people to work with.

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To my parents, Carmen and Luis and my brother Luis, to whom I owe everything I am.

1.Introduction

1.1. Human immunodeficiency virus (HIV)

1.1.1. Origin and transmission

HIV was isolated for the first time from a lymph node of an individual with marked lymphadenopathy in 1983 [2]. It was then identified as the causal agent of the acquired immunodeficiency syndrome (AIDS), a condition first described in 1981 [3]. From simian origin, the simian immunodeficiency virus, SIV, first jumped species in the 1920s in Kinshasa (currently, Democratic Republic of Congo) [4]. HIV is a lentivirus (a subclass of retrovirus) that contains between zero and ten copies [5] of single-stranded RNA.

Viral exposure at mucosal surfaces (by sexual contact or breastfeeding) as well as percutaneous inoculation (infected cutting materials or needles) or vertical transmission during pregnancy or delivery (mother to child transmission) compose the principal transmission routes of the virus [6]. It exists as two different viral strains, HIV-1 and HIV-2. HIV-1 is the most common one, responsible for 95% of the infections worldwide **(figure 1).**

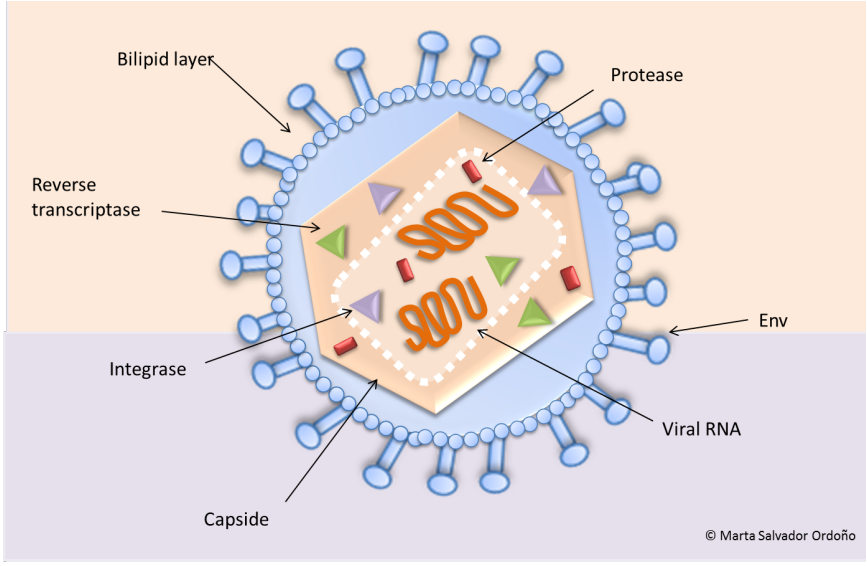

Figure 1. Structure of a mature HIV virion.

1.1.2. Epidemiology

Since the start of antiretroviral treatments in 1987, the incidence of new HIV cases and especially the number of deaths related to AIDS complications has drastically been reduced [7] . This is not only due to the expansion of the antiretrovirals (ARVs) but also a the result of implementing of prevention programs, which promote safer sex as well as prevention of mother to child transmission (MTCT) [7]. Consequently, the number of people living with HIV has increased.

Nonetheless, HIV infection is still a major concern, especially in resource-limited countries where 70% of the infection burden is located [7]. Globally, in 2015, there were over 2.1 million new HIV diagnoses, 1.1 million deaths from AIDS-related illnesses, and 36.7 million people living with HIV according to the Joint United Nations HIV/AIDS program, UNAIDS[8].

1.1.3. Replication cycle

HIV's life cycle consists of seven main steps: binding or attachment, fusion, reverse transcription, integration, replication, assembly, and budding and maturation **(figure 2).**

a) Binding

This is the first and most critical step of the viral replication process, its success will determine the efficiency of the infection. It begins with the adhesion of the infectious viral particle to the host cell surface by binding to the CD4 receptor and co-receptors [9]. This binding triggers conformational changes in the virus that will facilitate the following step, membrane fusion, and successive viral entry.

b) Fusion

In this step, the viral envelope fuses with the cell membrane, the viral capsid uncoats, and the viral genetic material gets released into the cytoplasm of the host cell along with the viral enzymes [9].

c) Reverse transcription

The virus carries an enzyme, reverse transcriptase (RT) which reverse-transcribes viral RNA (single-stranded) onto DNA (double-stranded) [10]. Following this, the pre-integration complex consisting of viral and host proteins as well as viral DNA translocates into the cellular nucleus through the nuclear complex pore. This step enables HIV to infect CD4 T cells, as well as other types of cells such as microglial cells or macrophages [11].

d) Integration

Following the nuclear entry, the reverse transcribed DNA gets incorporated into the host's DNA [12] via covalent attachment in a process similar to the DNA transposition [13]. The integration of the viral DNA is mediated by the viral-encoded integrase enzyme [14]. This multi-step process occurs in several steps. firstly removal of the terminal dinucleotides from each 3' ends and secondly, anneal –covalent bonds- to the target DNA (host's) [5].

The stages in which the integration occurs are as follows:

1. Formation of a complex with specific DNA sequences at each end of the HIV-1 long terminal repeat region.

2. Removal of a pair of nucleotides from each 3' end of the viral DNA.
3. Transfer of the viral DNA strand by covalent link (from the 3' ends) to the host's (target) DNA.

To date, there is no known human analog to the viral integrase enzyme; as such it is an interesting target for new antiretrovirals [15].

e) Replication

Once the proviral DNA is integrated into the host's DNA it is transcribed along with it, as any other cellular gene, upon every replication cycle during cell division [16]. The proviral genome (approximately 9 kb) is translated into a polypeptide which is then cleaved by the viral-encoded protease for the virus to mature. The proviral genome encodes for 9 proteins: 3 structural proteins (Gag, Pol, Env), 2 regulatory proteins (Tat, Rev) and 4 accessory proteins (Nef, Vif, Vpu, and Vpr)[2]. It will yield the necessary structural polypeptides and enzymes, for the successful production of new virion particles. [13]

f) Assembly

Assembly is an essential step for the spread of the infection to susceptible host cells. The essential viral components (viral structural and enzymatic proteins) previously synthesized are then packed forming new immature (non-infectious) viruses [17]. The newly synthesized particles travel to the plasma membrane, forming a spherical particle with a lipid cover (a portion of the host cell membrane), the viral RNA gets encapsidated and the viral envelope proteins spike, facing outwards as it buds the cell to infect new cells. [18]

g) Budding and maturation

Newly assembled but immature viruses cross the plasma membrane (in a cytoplasm-membrane vesicle) and undergo major structural changes in a step known as maturation [18]. The maturation is relatively quick since it is believed to be completed a few minutes after the virions leave the cell [19]. This is a needed step for the virions to become infectious.By this mechanism, the virus exploits the host's cell machinery to produce new copies of its genome and spread the infection.

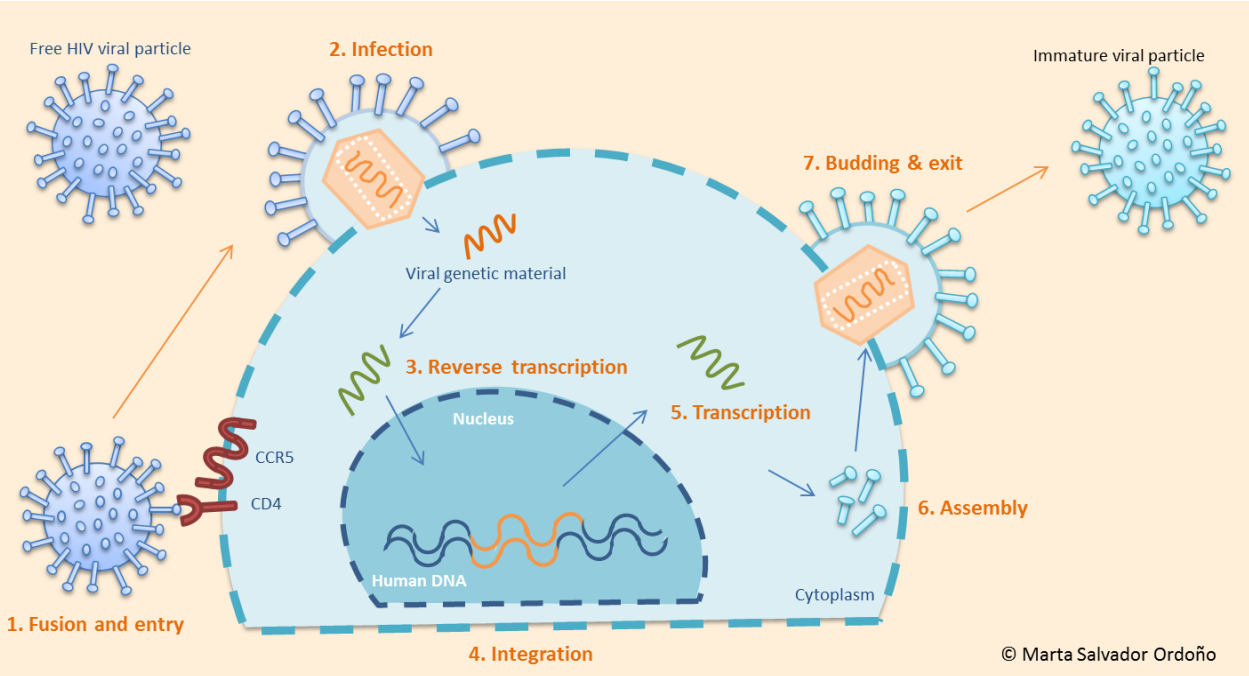

Figure 2. Scheme of the HIV replication cycle.

1.1.4. Pathogenesis and stages of the infection

The HIV infection starts with a progressive decrease in the number of CD4 T lymphocytes, weakening the immune system until opportunistic infections make their appearance. The rate at which the infection will progress varies between individuals, but on average, if the person

remains untreated, AIDS will develop within 10 years [20]. More rarely, some individuals will remain asymptomatic even without treatment. They are considered *long-term non-progressors or elite controllers*, and this occurs in less than 5% of the total HIV population [21]. Another group, categorized as *rapid progressors* develop AIDS within 3 years if untreated. They represent approximately, the 10% of the HIV population [22].

The different stages of the HIV infection include acute infection, chronic infection (clinically latent) and AIDS and their characteristics are described as follows:

i. Acute infection

At this stage, the individual looks healthy but the virus is actively replicating and disseminating in their bloodstream and lymph nodes (causing lymphadenopathy). It varies greatly between individuals but, in general, this stage might come with no symptoms or then unspecific cold-like symptoms. During the acute phase of the infection, the individual will undergo seroconversion (HIV antibodies will be detectable in blood) [23]. The timeframe varies from 1-6 weeks, up to 6 months [24].

ii. Chronic infection

This stage might persist for several years during which time, CD4 cell count progressively decreases [23]. The rate of disease progression varies hugely among individuals.

iii. Acquired Immune Deficiency Syndrome (AIDS)

This is the ultimate stage of the infection. It is characterized by immunosuppression via marked depletion of CD4+ T cells as viral load increases simultaneously. Once the CD4+

T cells count is lower than 200 cells/mL, reactivation of viral infections and opportunistic infections occur [25].

1.1.5. HIV and pregnancy

1.1.5.1. Overview

Mother-to-child transmission (MTCT), accounted for over 90% of the new infections among children younger than 15 years old in 2012 [26]. Pregnant women living with HIV can transmit the infection to their child (vertical transmission) by three main routes. The infection can be passed prenatally through the placenta especially toward the end of pregnancy. It can also occur perinatally, either during labor or delivery (both vaginal and via cesarean section), and in the postnatal period via breastfeeding [27]. The risk of transmission raises as the maternal viral load increases, which makes it important to treat with ARVs during pregnancy. Due to the spread of the ARV treatment among pregnant women as well as the implementation of preventive measures to improve HIV prenatal care, counseling, and testing, the percentage of children newly infected with HIV has decreased approximately 70% from 2000 to 2015 according to the 2016 UNAIDS Children and HIV informs [28].

In Europe and the United States, the rate of MTCT is now lower than 2% [29]; and lower than 1% in Canada [29]. Despite the success of MTCT prevention, in 2013 there were 3.2 million children living with HIV [30], and it was estimated in 2015 that approximately 150 000 perinatal infections still occur each year, an important decrease, from the estimated 490 000 perinatal MTCT in 2000 [28].

1.1.5.1.1. Prevention of mother-to-child-transmission

MTCT, also known as vertical transmission, is the most common way that children become infected with HIV [31]. Most of the MTCT occurs late in pregnancy or during delivery [32]. In the absence of intervention or treatment, the vertical transmission rate has been estimated between 14 and 48% [27] [33]. To improve the health of people living with HIV, current guidelines recommend starting lifelong antiretroviral treatment as soon as diagnosed, this also contributes to preventing MTCT in pregnant women living with HIV.

Previous guidelines recommended initiating treatment after the 10th or 12th week of gestation, once the fetus's organs have mostly developed, in order to reduce side effects including malformation of the developing fetus [34], unless maternal CD4 count dictated treatment according to the standard of care. In some instances (i.e uncontrolled or unknown viremia near the end of the pregnancy) pregnant women living with HIV are scheduled for a cesarean delivery(C-section, C/S) to prevent MTCT during delivery [35].Furthermore, infants born to women living with HIV will receive ARV (usually Zidovudine) for 4 to 6 weeks after birth as a prophylaxis measure [36]. Because HIV can be transmitted through breast milk, women with HIV living in North America are recommended not to breastfeed their newborns. All these preventive measurements have greatly reduced the MTCT rate approximately an 80% in the last 20 years in the North America [37]

1.1.5.2. Pregnancy outcomes for women living with HIV

Negative pregnancy outcomes include preterm birth (PTB), low birth weight, spontaneous abortion, stillbirth, fetal abnormality, intrauterine growth restriction (IUGR), and

perinatal, neonatal or infant mortality among others[38].A meta-analysis of studies on maternal HIV and pregnancy outcomes published between 1983 to 1996 identified 31 prospective studies (10 studies were in developed countries; 21 studies were in developing countries). In general, the meta-analyses concluded that pregnant women living with HIV were at a greater risk (odds ratio, ranging from 4.05 to 1.10) for adverse pregnancy outcomes [39]. The mechanisms underlying these negative outcomes remain unclear.

One possible explanation might be an adverse effect of the ARVs. Some ARVs are known to cross the placenta, as well as the blood-brain barrier [40][41]. More specifically protease inhibitors, have been associated with adverse birth outcomes such as an increased risk of premature delivery as well as infant low birth weight and fetal growth restriction [42][43][44][45]. It is estimated globally that more than 13 million births occur annually (11% of live births) are preterm births (PTB) majority of them, in low-income countries[46]. However, among women living with HIV (treated or not) the rates of PTB are much higher (ranging from 18% to 27% [47]).

There remains some controversy regarding the effects of ARVs on pregnancy and infant outcomes [48]. Some studies have shown associations between pregnancy complication and negative birth outcomes (PTB, small for gestational age, IUGR, stillbirths, and preeclampsia) and some ARV treatments [49] [50] or the infection itself [48] [51]. However, other studies have not found any significant associations [52] [53] [54] that link increased the risk of negative birth outcomes and treatment with ARVs. Due to the lack of consistency in this regard, more research on this topic is needed to elucidate the impact of the ARVs on pregnancy outcomes and assess, which are safer during pregnancy.

1.2. Combination Antiretroviral Therapy (cART)

With the advent of ARVs in the early 90s, HIV infection has become a manageable chronic condition rather than a deadly disease, as it was before the "combination antiretroviral therapy (cART) era". In 1987, the FDA approved the first HIV antiretroviral drug (Zidovudine, AZT). Before the cART era, AZT, as well as the successively discovered ARVs (Didanosine, ddI, in 1991 and Zalcitabine, ddC, in 1992), was given as monotherapy. As new drugs were discovered, ARVs started to be given as a combination of two, referred to as "dual therapy". The purpose was to reduce the development of drug resistant-HIV strains as well as to increase the effectiveness of the treatment [55]. Since 1996, ARVs have been given in a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) –also known as "backbone"- along with one non- nucleoside reverse transcriptase inhibitors, protease inhibitors or integrase strand transfer inhibitors – also known as the base.

Regarding the timing of initiation of HIV treatment, the guidelines have evolved over time and between countries due to the different resources [20]. Initially (1997), treatment initiation was guided based on the CD4 count and the viral load (i.e. "treatment should be offered to individuals with fewer than 500 CD4+ T cells/mm3 or plasma HIV RNA levels exceeding 10 000 copies/mL") [56]. Current guidelines recommend starting treatment at diagnosis, regardless of the CD4 count or viral load, in order to decrease morbidity associated with HIV, increase life expectancy, and reduce transmission risk [57] .

Overall, treatment has reduced HIV-associated morbidity and mortality, contributed to prolonging life expectancy, prevented transmission and most importantly, it has increased the

quality of life for people living with HIV. Given the growing number of people treated with ARVs, there is an increasing concern about the side effects and long-term consequences of chronic use of these medications. Some ARVs have been known to cause life-threatening adverse side effect [58] [59] [60]. Nevertheless, the positive effects of the ARVs treatment outweigh the potential adverse outcomes, especially for pregnant women. Consequently, it is important to determine the safety of ARV regimens during pregnancy to inform guidelines for women living with HIV.

1.2.1. cART types

To date, there are in total 25 ARVs, grouped in 5 classes, (nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTI), entry and fusion inhibitors (EI and FI) -chemokine receptor antagonists-). They act by inhibiting or interfering with different steps of the HIV cycle (viral entry, viral reverse transcriptase, nuclear integration, viral proteins assembly).

1.2.1.1. Nucleoside reverse transcriptase inhibitors (NRTIs)

NRTIs are prodrugs that mimic the structure of nucleosides but are missing the 3'OH group. NRTIs are pharmacologically inactive until phosphorylated intracellularly by cellular kinases [10]. These medications interfere with the viral reverse transcriptase (RT) enzyme competing with endogenous nucleotides to be incorporated into the viral DNA, and therefore, inhibit viral replication through chain termination.

Over the past 15 years, several studies –in humans as well as in animals- reported mitochondrial toxicity with NRTI treatment [61]. NRTIs can inhibit the mitochondrial enzyme polymerase © -responsible for mtDNA replication and repairation- [62][63]. Regarding their ability to cross the placenta, NRTIs have been shown to reach relatively high pharmaceutical concentrations in the fetal circulation [64] giving rise to concern about the side effects that NRTIs might have on the developing fetus. To date, 7 NRTIs have been approved by FDA **(table 1)**

Table 1. Nucleoside reverse transcriptase inhibitors brand, generic and abbreviated names and approval dates

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)			
Brand Name	**Generic Name(s)**	**Abbreviated Name**	**Approval date**
Retrovir	Zidovudine, Azidothymidine	AZT	Mar-1987
Videx	Didanosine, Dideoxyinosine	ddI	Oct-1991
Hivid	Zalcitabine	ddc	Jun-1992
Zerit	Stavudine	d4T	Jun-1994
Epivir	Lamivudine	3TC	Nov-1995
Combivir	Lamivudine, Zidovudine		Sep-1997
Ziagen	Abacavir Sulfate	ABC	Dec-1998
Videx EC	Enteric coated didanosine	ddI EC	Oct-2000
Viread	Tenofovir disoproxil fumarate	TDF	Oct-2001
Emtriva	Emtricitabine	FTC	Jul-2003

1.2.1.2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

This class of ARVs acts binding non-competitively to the active site of the viral RT. Compounds of this class inhibits RT by blocking its DNA polymerase activity [10]. **(Table 2)**

Table 2. Non-nucleoside reverse transcriptase inhibitors brand, generic and abbreviated names and approval dates

Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
Brand Name	Generic Name(s)	Abbreviated Name	Approval date
Viramune	Nevirapine	NVP	Jun-1996
Rescriptor	Delavirdine	DLV	Apr-1997
Sustiva	Efavirenz	EFV	Sep-1998
Intelence	Etravirine	ETR	Jan-2008
Edurant	Rilpivirine	RPV	May-2011

1.2.1.3. Protease inhibitors (PIs)

PIs inhibit the proteolytic activity of the viral protease (an essential viral enzyme, responsible for the production of mature and infective virions) [65]. PIs have shown poor transfer across the placenta [64] but it is known that some PIs inhibit the cytochrome P450 (CYP3A4) [66] **(Table 3).**

Table 3. Protease inhibitor brand, generic and abbreviated names and approval dates

Protease Inhibitors (PIs)			
Brand Name	Generic Name(s)	Abbreviated Name	Approval date
Invirase	Saquinavir	SQV	Dec-1995
Norvir	Ritonavir	RTV	Mar-1996
Crixivan	Indinavir	IDV	Mar-1996
Viracept	Nelfinavir	NFV	Mar-1997
Agenerase	Amprenavir	APV	Apr-1999
Kaletra	Lopinavir, Ritonavir	LPV/RTV	Sep-2000
Reyataz	Atazanavir	ATV	Jun-2003
Lexiva	Fosamprenavir	FOS	Oct-2003
Aptivus	Tipranavir	TPV	Jun-2005
Prezista	Darunavir	DRV	Jun-2006

1.2.1.4. Integrase strand transfer inhibitors (INSTI)

This is the newest class of ARVs. INSTIs act by blocking the integration of the proviral DNA into the host genome, through inhibition of the viral enzyme *integrase* [14] **(Table 4)**. The first INSTI on the market was Raltegravir, approved by the FDA in 2007 but it is with the advent of more recent drugs, (dolutegravir) that this class has become commonly used.

Table 4. Integrase strand transfer inhibitors brand, generic and abbreviated names and approval dates

Integrase Strand Transfer Inhibitors (InSTIs)			
Brand Name	Generic Name(s)	Abbreviated Name	Approval date
Isentress	Raltegravir	RAL	Oct-2007
Tivicay	Dolutegravir	DTG	Aug-2013
Vitekta	Elvitegravir	EVG	Sep-2014

1.2.1.5. Entry and fusion inhibitors (EI and FI)

Also known as chemokine receptor antagonists -CCR5 antagonists-, this family of ARVs are the newest ARVs (the first one of its class, Maraviroc, was approved by the FDA in 2007) and act by blocking the fusion and entry of the virus via allosteric binding to the CCR5 receptor on the cellular membrane of CD4+ T cells [67] When the CCR5 receptor is unavailable, the virus cannot enter the cell, therefore these ARV decrease viral infectiousness [67]. Due to the lack of clinical data regarding their safety and possible teratogenicity, these ARVs are rarely prescribed in pregnancy [68].

Table 5. Entry and fusion inhibitors brand, generic and abbreviated names and approval dates

Entry and Fusion Inhibitors			
Brand Name	Generic Name(s)	Abbreviated Name	Approval date
Fuzeon	Enfuvirtide	T-20	Mar-2003
Selzentry	Maraviroc	MVC	Aug-2007

1.2.2. cART guidelines for pregnancy

The standard of care for pregnant women living with HIV is cART, but with the increasing number of ARVs, the selection of the safest treatment is gaining importance. In numerous clinical cases, regimens that were not well studied in pregnancy were prescribed to pregnant women living with HIV. This emphasizes the need for research to investigate the safety of all ARVs during pregnancy.

Previous guidelines for pregnant women living with HIV recommended starting cART in the 2nd trimester. Current Canadian guidelines recommend initiating treatment as soon as diagnosed with HIV, regardless of CD4 cell count at baseline as well as HIV viral load. In addition, experts recommend not stopping or changing ARVs treatment during the first trimester for obstetrical reasons. If for any reason during pregnancy, cART was discontinued, the guidelines suggest discontinuing all drugs at once to minimize the risk of developing viral resistance. cART should be started again as soon as possible in order to avoid the rebound of the viral load as well as to avoid possible MTCT. It is also recommended for all women living with to get their virus genotyped (as well as phenotypic resistance testing, if possible) in order to optimize cART.

Regarding the mode of delivery, experts distinguish two situations: for those women with optimal adherence to cART and undetectable or low viral load (less than 1,000 c/ml) in the last 4 weeks before the delivery, is recommended to have a vaginal delivery. On the other hand, for women with poor adherence to cART and/or high (not suppressed) viral load, a scheduled caesarian section (C/S) is recommended after 38 weeks of gestation [69]

Administration of AZT intravenously should be initiated from the onset of the labor and until delivery, regardless of the mode of delivery and viral load, as a measure to prevent MTCT. For women living with HIV, the recommendation is to receive 2 NRTIs (AZT+3TC, ABC+3TC, TDF+ FTC or TDF+3TC) combined with a 3rd drug (a boosted-PI, an INSTI or a NNRTI, such as LPVr, RAL or EFV). DTG is the newest INSTI but there is not yet, much information regarding its teratogenicity neither its possible effects on pregnancy outcomes [70].

When possible, EFV should be avoided in the first trimester of pregnancy due to its possible teratogenicity, but if the mother was on EFV before pregnancy, and the timing is beyond when the neural tube closure occurs, the treatment with EFV could be continued. In addition, NVP should not be started in pregnancy unless required based on resistance patterns due to the high rate of serious adverse outcomes. All those recommendations among others appear in the latest British Columbian guidelines to prevent MTCT [69,71].

1.3. Preterm Birth

1.3.1. Overview

Preterm birth (PTB), defined as infants born at <37 weeks of gestation, is the leading cause of death and morbidity among children younger than age 5 years and was responsible for more than 1 million deaths worldwide in 2015 [1]. PTB occurs in 6–10% of births among the Canadian population [72], however among pregnant women living with HIV, the rates are much higher, ranging from 18 to 29% [73] [74]. To date, there is no generally accepted mechanism underlying such increased rates.

PTB can be medically prescribed by the physician for maternal or fetal indications, in which case labor will be induced or the infant is prematurely delivered via C/S (before labour). Such PTB is known as *iatrogenic.* PTB can also happen spontaneously, or be a preterm with premature rupture of membranes (PPROM), irrespective of the delivery. For clarification purposes, births due to spontaneous labor as well as PPROM are both considered *spontaneous* PTBs [75]. Thirty-one to 50% of the PTBs occur spontaneously, 12 to 28% are derived from a multiple pregnancy (and associated complications), 6 to 40% occur with PPROM 12% have preeclampsia as the etiology and IUGR is responsible for 2 to 4% of PTB [76].

1.3.2. Factors associated with PTB

To date, there is no clear and single cause of PTB and it is rather regarded as multifactorial. Nonetheless, some factors have shown association to increased risk of premature delivery:

- Maternal age [77] (being younger than 18 and older than 45 years)
- Black ethnicity [76] [75] [78]

- Poor socioeconomic conditions [76]
- Substance use (smoking, drugs, alcohol) [79]
- Multiple pregnancies (i.e. twins) [75]
- History of previous of PTB [75] [78]
- Overweight or underweight pre-pregnancy [80] [81]

Some pregnancy pathologies are also frequently associated with PTB:

- Preeclampsia (high blood pressure and proteinuria) [78]
- Intrauterine growth restriction (IUGR) [75]

One possible explanation behind the *spontaneous* PTBs in the context of HIV is the reduced maternal progesterone production during pregnancy, which may be related to HIV infection and/or antiretroviral treatment. In the general population alteration on the progesterone receptor has been associated with induction of labor [82].

1.3.2.1. Pregnancy complications

1.3.2.1.1. Preeclampsia

Preeclampsia is defined as gestational hypertension (develops for the first time at or after the 20th week of gestation) with one or more of the following: new onset **proteinuria** (presence of proteins in the urine, > 0.3 g per 24 hours) or one or more **adverse conditions** or **severe complications** (i.e. renal insufficiency, thrombocytopenia, impaired liver function, pulmonary edema, or cerebral or visual problems [83]) , according to the Canadian guidelines elaborated in

2014 by the Society of Obstetricians and Gynecologists of Canada [84]. In 2014, preeclampsia was the leading cause of maternal mortality and severe long-term morbidity worldwide [85]. It has been proposed that preeclampsia results from defective spiral artery remodeling which leads to cellular ischemia in the placenta [83]. Preeclampsia is more common among women with: a multiple pregnancy, history of diabetes mellitus or hypertension, nulliparity, pre-existing hypertension, or obesity (pregestational BMI $\geq$30kg/m^2) [85]. The rates of preeclampsia worldwide vary from 5 to 8% [83], but in Canada, the rate is 4.5%, and in British Columbia the rate is 4% [86]. Among the HIV+ women in our cohort, preeclampsia rates are slightly higher than 7% (14 cases out of 196 study participants).

Depending on the emergence of the preeclampsia symptoms, physicians distinguish between two subtypes of preeclampsia: early (before the 34 weeks of gestation) and late (after the 34 weeks of gestation) onset preeclampsia. The late-onset subtype accounts for the majority of the cases of preeclampsia (>80%). Nevertheless, the early onset preeclampsia is associated with most of the maternal and fetal mortality and morbidity rates [83]. If preeclampsia remains untreated it can lead to maternal eclampsia, which is characterized by seizures or death [85].

The cause of late onset preeclampsia is probabaly often due to maternal predisposition rather than associated with placental pathology, in contrast, the cause of early onset preeclampsia is initially, poor remodeling of maternal uterine arteries what leads to poor blood perfusion to the placenta, and this leads to placental dysfunction, likely derived from damaged placental mitochondria [87] [88], hypoxia and reduced nutrients supply to the growing fetus, which could lead to *intrauterine* growth restriction, IUGR[83]. It has been suggested that IUGR and

preeclampsia may share a common pathway possibly explained by placental insufficiency [89]: a pregnancy complication characterized by the inability of the placenta to deliver an adequate supply of nutrients and oxygen to the fetus and therefore, it cannot fully support the developing fetus, it is often associated with dysfunctional mitochondria unable to meet the energy demands required early in gestation. This process requires high availability of energy for cell growth, proliferation, and metabolic activity. Insufficient energy supply would possibly result in placental insufficiency [90]. is a complication of pregnancy when the placenta is unable to provide an adequate supply of nutrients and oxygen to the fetus, and, thus, cannot fully support the developing baby.

1.3.2.1.2. Intrauterine growth restriction

Intrauterine growth restriction (IUGR) is a pathological decrease in the rate at which a developing fetus grows. This multifactorial condition contributes to infant morbidity and it is associated with long-term conditions such as impaired growth during childhood, as well as metabolic syndrome (defined as the cluster of risk factors such as obesity, poor maternal nutrition, smoking or alcohol use, infections, chromosomal aneuploidies,insulin resistance, hypertension and dyslipidemia that will increase the odds to develop type 2 diabetes mellitus and cardiovascular diseases [91] [92]) respiratory complications and neurological impairment (i.e cerebral palsy [93]) [94] [48]. In terms of its epidemiology, it is estimated that 3 to 7% of the infants are born with IUGR [95] worldwide.

As consequence of this restricted growth, most infants are born “small for gestational age”, SGA. Nevertheless, not all the SGA newborns will be born with IUGR; as some newborns

are simply constitutionally small, not related to any pathology. Only those infants whose birth weight and/or length is below the 10th percentile for their gestational age will be considered SGA infants [96] Nonetheless, all the newborns that are IUGR will be SGA if using the beforementioned birth measurement. Some well-established risk factors of IUGR are maternal smoking, placental dysfunction, gestational hypertension, PE, poor nutrition, low or high maternal body index mass (BMI) and high maternal stress level among other factors. [95].

1.3.2.1.3. Gestational diabetes mellitus

Gestational diabetes mellitus, GDM, is the most common pregnancy-specific condition. It is characterized by glucose intolerance (any degree) with onset or first recognition during pregnancy. This definition of GDM does not exclude the possibility that the glucose intolerance may have started before pregnancy and it applies whether or not the patient is under insulin treatment[97] . This metabolic disorder occurs in approximately 6% of pregnancies according .to the Public Health Agency of Canada[98]and is associated with negative maternal and neonatal outcomes [99] (such as increased risk of macrosomia –birth weight > 4000 g regardless of the newborn's gestational age-- [100]. Some studies also found association between GDM and negative pregnancy outcomes including preeclampsia, C/S [101], congenital abnormalities [102] and perinatal death [103]. Risk factors for GDM are obesity, family history of diabetes, older maternal age as well as gestational hypertension [104]

The physiopathology behind GDM is a progressive decrease in insulin sensitivity and therefore, relative glucose intolerance. Insulin-resistance arises around the 2nd trimester and remains until the delivery. Furthermore, the increased production of hormones (cortisol, progesterone, and estrogen) during pregnancy also contributes to an altered insulin-glucose

balance. To compensate for the insulin resistance, the pancreas secretes higher levels of insulin. GDM occurs in those pregnancies when the women's pancreas cannot secrete enough insulin to overcome the insulin resistance [104].

1.4. Progesterone

1.4.1. Function and synthesis

Progesterone, colloquially known as the pregnancy hormone, is a "pro-gestational" hormone. From a biochemical point of view, progesterone is a steroid gonadal hormone endogenously synthesized in the corpus luteum (ovaries) during normal female ovulation. After fertilization and implantation, the corpus luteum continues producing and secreting progesterone what prevents menstruation and provides the adequate environment for the developing embryo [105]. After approximately the 10th week of gestation, its production is taken over exclusively by the placenta which continues up to the end of the gestation, at which time the levels drop to indicate the end of the gestation and initiation of parturition [106]. Progesterone is rapidly metabolized in the liver and has a half-life of approximately five minutes in circulation [107].

Progesterone is essentially required by all mammals for the survival and development of the fetus [108]. It has a central role in maintaining the pregnancy by suppressing the calcium–myosin kinase system that provides uterine quiescence and achieves the resting state of the uterine smooth muscle [82]. It is also known that progesterone has anti-inflammatory properties which is of great importance in the context of HIV, where the patients deal with a chronic state of inflammation [109]. Progesterone is also a central element for the synthesis of other sex hormones as well as corticosteroids and it has protective effect in the brain by mediating

myelination [110]. Among nonpregnant women, progesterone regulates menstruation and ovulation, in pregnant women it regulates embryogenesis and fetus development [111]. Given its protective role in pregnancy, in 2011 FDA approved the supplementation of progesterone (hydroxyprogesterone caproate) during pregnancy to reduce the risk of preterm deliveries in women with a history of prior spontaneous PTB [112].

The synthesis of progesterone occurs in a two-step reaction. It is derived from maternal circulating cholesterol, which is converted to pregnenolone by *cytochrome P450 side chain cleavage (scc)* (CYP11A1). Pregnenolone is then converted to progesterone by *3beta-hydroxysteroid-dehydrogenase*, 3® HSD. Both enzymes are located on the inner mitochondrial membrane. Synthesis of progesterone, which is central to pregnancy maintenance, is therefore dependent on placental mitochondrial function [113] and the transport of cholesterol from the cellular cytoplasm to the inner mitochondrial membrane, the rate limiting step of the reaction [114] **(Figure 3).**

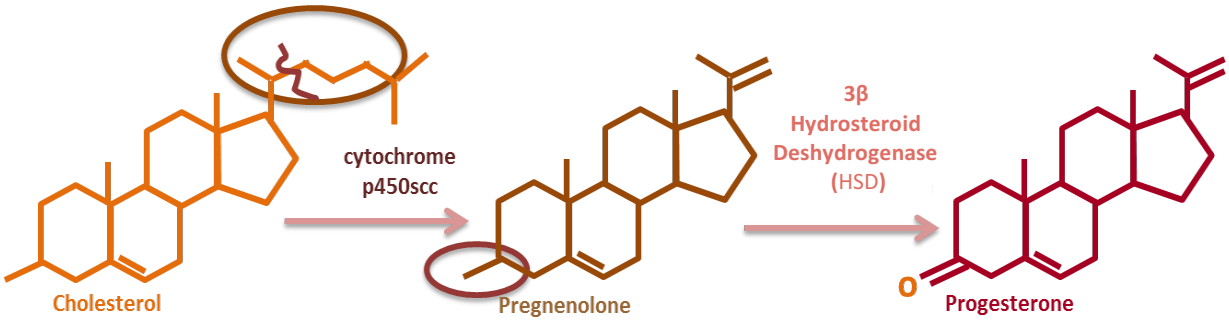

Figure 3. Synthesis of progesterone from maternal circulating cholesterol

Importantly, some PIs (especially Ritonavir) are known to strongly bind to the CYP11A1, which could lead to an inhibition of its enzymatic activity (ranging from a weak inhibition for

Saquinavir to very potent inhibition for Ritonavir) [20] [29] [115] [116]. As shown above in **figure 3** CYP11A1plays a central role in the synthesis of progesterone, consequently, if the enzyme is inhibited, the production of progesterone will be impaired [117]. Also, a recent Canadian study has reported greater expression of 20 alpha hydroxysteroid dehydrogenase (20 α HSD) in placenta from women living with HIV and exposed to PI-based cART compared to HIV uninfected women [118]. This enzyme converts progesterone into a metabolically inactive product, (20α- hydroxyprogesterone) and in doing so, it plays an essential role in the termination of pregnancy and the initiation of parturition [119]. This proposed explanation could be one of the mechanisms behind the lowered levels of plasma progesterone (measured on maternal blood samples 24-28 weeks of gestation) found among the women living with HIV, compared to controls (HIV uninfected women) [118].

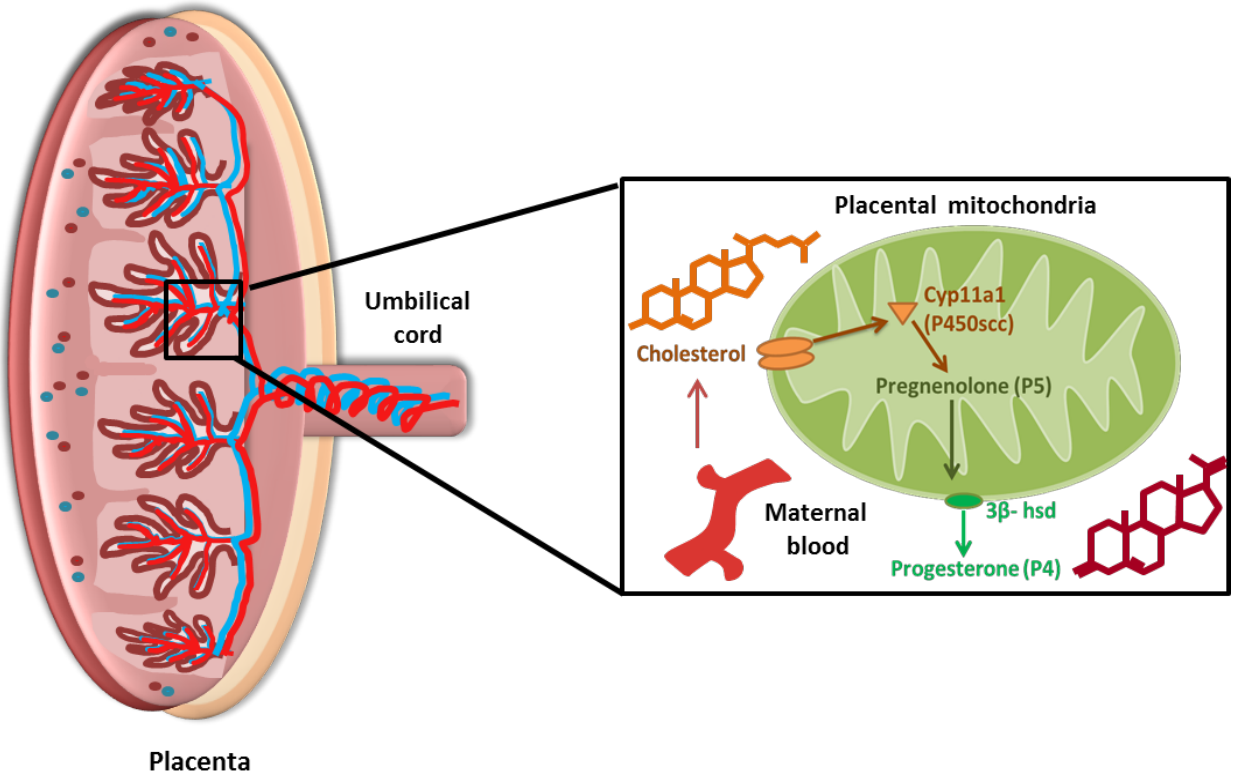

Figure 4. Schematic of the progesterone production in the placental mitochondria

1.4.2. Placenta

The placenta is the multifunctional and transient organ that allows the developing fetus to grow undisturbed by providing the necessary nutrients and oxygen, derived from the maternal circulation [120]. Highly vascularized, it is also responsible for the excretion of residual substances that would otherwise be toxic to the fetus. Moreover, another essential function of the placenta is the synthesis and secretion of different hormones, including progesterone, which is crucial during pregnancy [121] [122].

From a histological point of view **(figure 5)**, this highly specialized organ is composed of fetal tissues and is therefore considered a fetal organ, however it interacts with maternal tissues and blood vessels at the interface. [123]. On the maternal side, the **myometrium,** and the ***decidua basalis,*** are layers of tissue originated from maternal cells of the uterus wall and are therefore considered maternal tissue. Both remain in the mother when the placenta is delivered [124].After those two layers of maternal tissue, the placenta (mass of chorionic villi) is surrounded by extraembryonic membranes, the amniotic and chorionic membranes [123]. The chorionic membrane contains the chorionic villus. Each chorionic villus is separated from the contiguous, by the placenta septum and each of those units forms a cotyledon –separations of the *decidua basalis*-. Each villus is constituted by a layer of cytotrophoblasts and a layer of syncytiotrophoblast as well as an inner core containing fetal blood vessels, fibroblasts, macrophages, and other support cells. The cytotrophoblast (proliferative and mononuclear) either undergo cell division and become invasive, forming extravillous trophoblasts, or fuse to form the syncytiotrophoblast, (outer most layer of each villi, non-proliferative and multinucleated). Cytotrophoblast differentiation involves changes in the cell morphology, gain of the

steroidogenic activity (hormone production, *ie.* progesterone) and decrease of metabolic activity [113] [125]. All these successive cellular processes occur during early placentation (placenta starts to form after the second week of pregnancy) [126]. Despite their fetal origin, the trophoblasts (cyto and syncytio) do not trigger the maternal immune system [127]. Overall, placental cells are responsible for a variety of functions (attachment, invasion, hormones synthesis and nutrient/waste transportation and excretion) [113].

The placenta, therefore, plays a major role in the initiation and maintenance and successful evolution of the pregnancy. Unarguably, the optimal fetal growth depends on adequate placental function which, consequently, requires increased mitochondrial capacity to meet the energetic and metabolic needs of the growing fetus [128]. The placental mitochondrial function might, therefore, play an important role during the placentation as well as the full pregnancy. Possible mitochondrial dysfunction, induced by environmental factors, such as smoking or medical treatments or even maternal medical conditions developed during pregnancy such as preeclampsia may have detrimental effects on the developing fetus [128] Furthermore, placental inflammation or maternal infection can lead to placental complications [129] [83] .

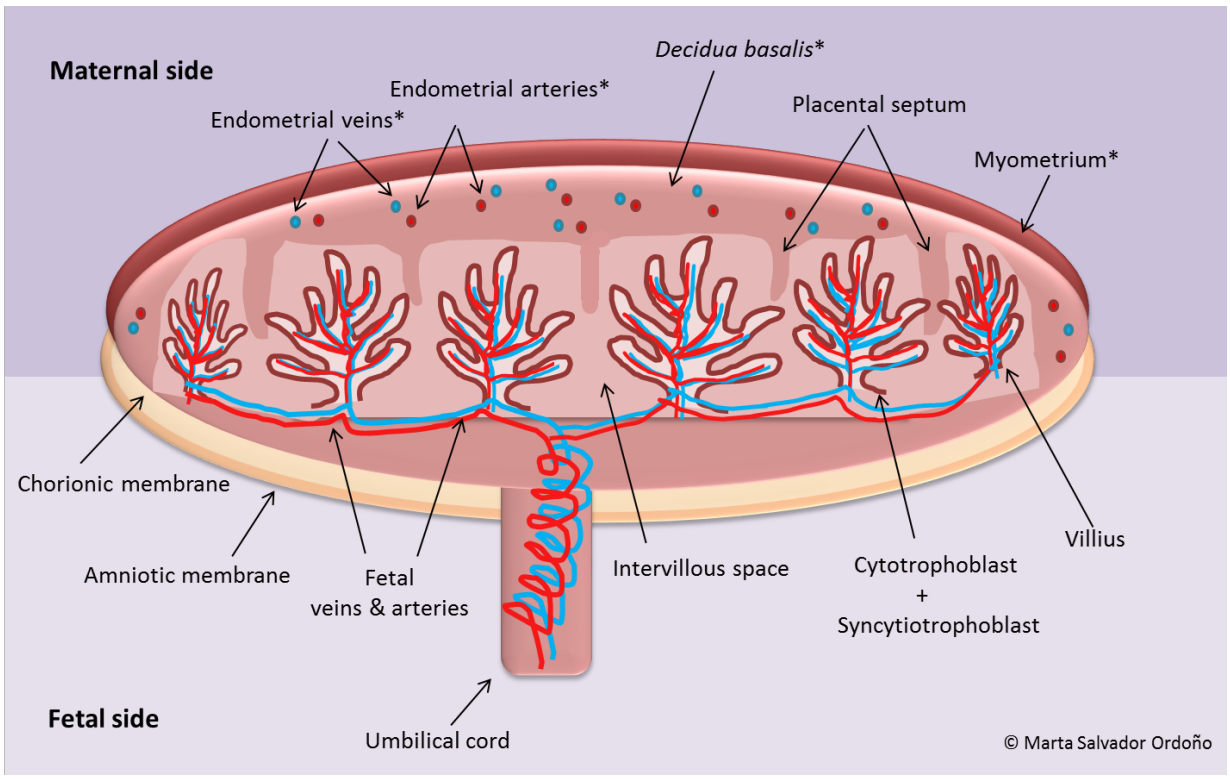

Figure 5. Schematic of the placenta. (*) denote maternal-origin tissue

1.5. Mitochondria

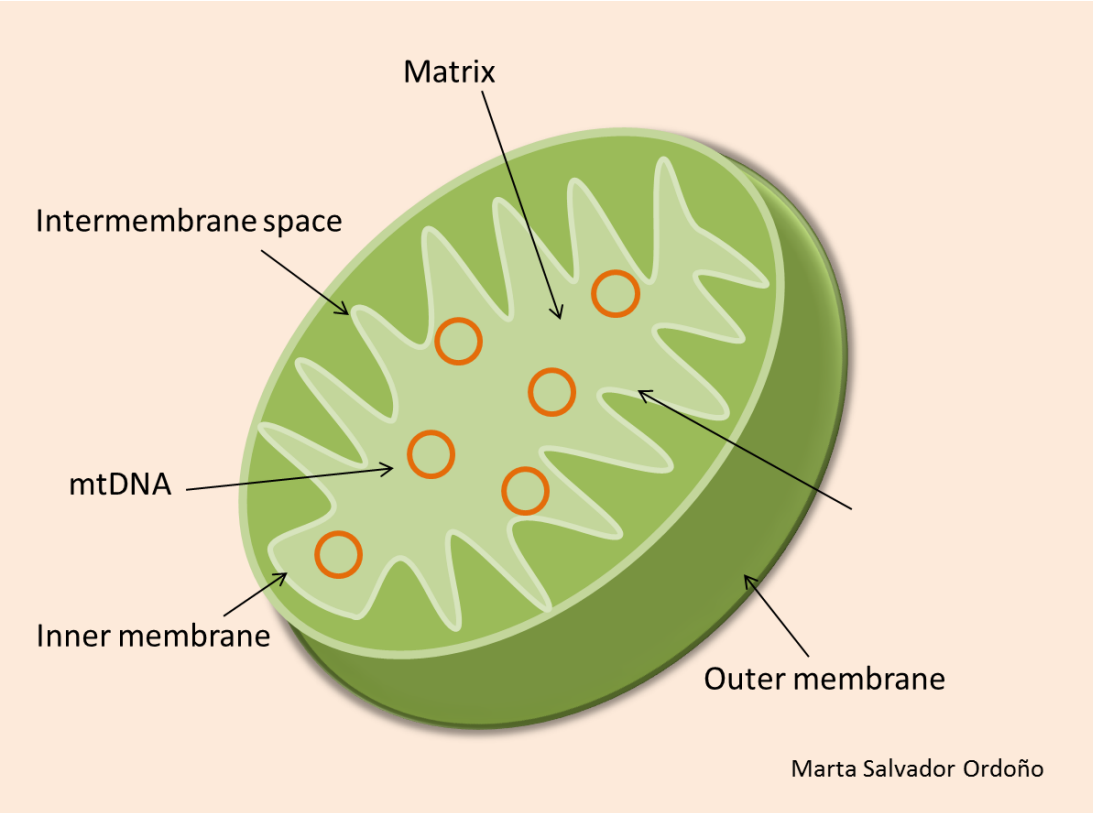

Figure 6 Schematic of a mitochondrion

1.5.1. Structure and function

Mitochondria are cellular organelles originated from archaebacterias –primitive unicellular bacterial microorganisms- [130] [131][132]. The *endosymbiotic theory* posits that the mitochondrion, was engulfed via phagocytosis by an anaerobic eukaryote [131]. However, alternative theory, the "pre-symbiont hypothesis", posits there was an already present metabolic organelle, "*the mitochondrion*" [130]. The real and true origin of the mitochondrion remains debated.

Mitochondria are known to be the cellular "powerhouse"[133] , given it is the main site of cellular ATP production. The mitochondrial matrix is where the citric acid cycle –also known as the Krebs cycle- occurs. It is, undoubtedly, the most efficient and important metabolic pathway. In that central biochemical chain reactions (8 steps), the cellular acetyl-CoA (common derivate from fatty acids, proteins, and carbohydrates) is oxidized and produces CO_2, H_2O, NADH, FADH, and ATP –the energy molecules that drive cell functions [134]. Exactly 12 ATP molecules are produced (directly and indirectly) with each cycle via oxidative phosphorylation (OXPHOS) [134].

Structurally, **(figure 6)** the mitochondrion has a double membrane (outer and inner membranes), which separates three main compartments: cellular cytoplasm, intermembrane space, and mitochondrial matrix. The outer membrane covers the organelle entirely and the inner membrane is folded creating the waved and layered structures known as the cristae [135]. The inner membrane is the site where the electron transport chain (ETC) is located **(figure 7).** The ETC is composed of 5 enzymatic complexes as well as 2 mobile electrons carriers (ubiquinone and cytochrome c) [136]:

1) NADH dehydrogenase (complex I)
2) Succinate dehydrogenase (complex II)
3) Ubiquinol cytochrome c oxidoreductase (complex III)
4) Cytochrome c oxidase (complex IV)
5) ATP synthetase (complex V)

Complexes I, III and IV pump H^+ (protons) to the intermembrane space, thus creating an electric gradient [133], that drives the synthesis of ATP by the ATP synthase. The electrons are driven from the NADH to the molecular oxygen, the final electron acceptor and which will be further reduced to H_2O [137]. Some other functions of the mitochondria are the synthesis of pyrimidines [137] (thymine and cytosine) and steroid hormones [111] (progesterone, testosterone); Ca^{+2} and Fe^{+2} homeostasis and apoptosis (programmed cell death) [138] [137].

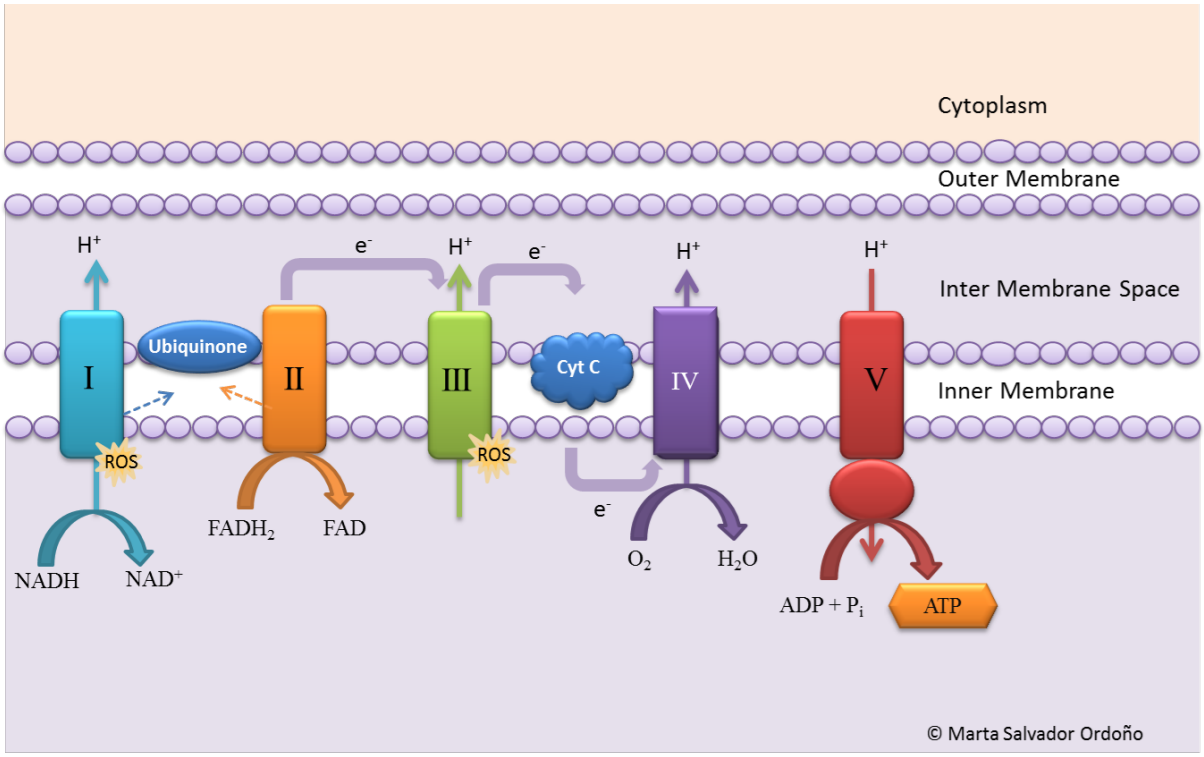

Figure 7. Mitochondrial electron transport chain.

1.5.2. Mitochondrial DNA (mtDNA)

Mitochondrial DNA is a circular double-stranded molecule of DNA present in the mitochondrial matrix, which includes 16 569 base pairs (bp) that codes 37 genes, of which all are essential for mitochondrial function. Out of those 37 genes, 13 genes encode for proteins

involved in the electron transport chain needed for mitochondrial respiration, 22 genes encode for transfer RNA(tRNA) and 2 genes code for ribosomal RNA (rRNA) [137] [139] **(figure 8)**. In contrast to the nuclear DNA, there are multiple copies of mtDNA within one cell, depending on the energetic requirement of the specific tissue, this can range from 10 to 10 000 copies [137].

MtDNA may be a good indicator of mitochondrial health and function [140]. Any defect in the mtDNA could affect the electron transport chain (ETC) and alter the production of energy – ATP-, and thus have a negative impact on the cellular functions [141].

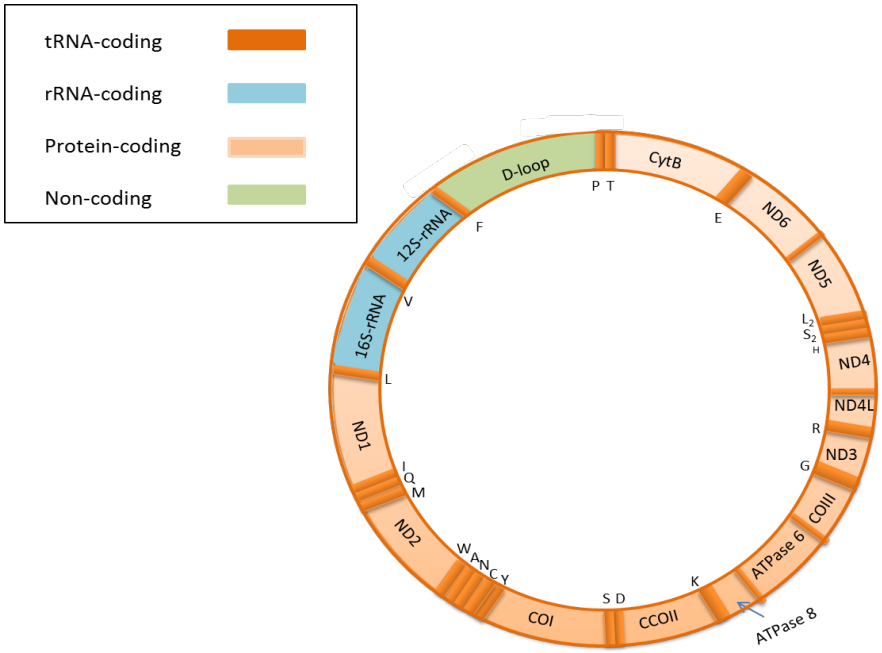

Figure 8. Mitochondrial double-stranded circular genome.

1.5.2.1. mtDNA replication and repair

Mitochondrial disorders are usually multisystemic although they affect tissues with higher energy demand such as skeletal muscle [139] [142]. Damage to mtDNA is linked to aging [135].

Given its location near the ETC, a major site of reactive oxygen species (ROS) production, mtDNA is more susceptible to oxidative damage than nuclear DNA (3 to 10 fold more) [139].

Mitochondrial division is primarily triggered either by a new cellular division or in response to an increase in energy demand on that specific tissue. Nevertheless, it could also be triggered in response to some other stimuli such as increased or decreased hormones levels, during development or even in some metabolic diseases. DNA pol © is the enzyme responsible for replication and gap-filling during mtDNA repairation [132]. This enzyme has polymerase, lyase, and 3′-5′ exonuclease activities [63] and along with several nuclear proteins, is involved in the replication of the mitochondrial genome. Unlike nuclear polymerases, pol © is prone to errors which partially explain the higher number of mutations seen in the mtDNA compared to nuclear DNA. The accumulation of these mutations over time might lead to mitochondrial dysfunction and tissue aging [143] [63]. Furthermore, low socioeconomic status has been reported in previous studies to be linked a higher incidence with chronic diseases, such as hypertension and diabetes, as well as higher incidence of preterm birth [1] [2].

1.6. Rationale, hypothesis, and specific aims

At least a decade of studies has suggested a link between cART and adverse pregnancy outcomes, including preeclampsia, preterm delivery, SGA infants, and lower birth weight, in the context of HIV [144] [49] [145]. Although *in utero* exposure to cART greatly reduces MTCT of HIV, previous studies have shown that women living with HIV on cART have 1.5-2.5 times increased the risk of preterm delivery [146] [44] [144]. cART is known to affect mitochondria,

and mitochondrial function is necessary for placenta growth and function, as well as for progesterone synthesis during pregnancy.

The aims of my thesis research are to:

1) Quantify mtDNA content (a marker of mitochondrial function) and progesterone levels in placenta tissue from both, HIVinfected and HIVuninfected women as well as PTB and TB.
2) Investigate possible associations between these measures and HIV infection, cART (type), and other relevant maternal factors or pregnancy complications, controlling for sociodemographic factors.
3) Explore whether PTB and the above measures are associated with HIV-specific clinical parameters among HIV+ women

I hypothesize that:

1) Placental mitochondrial dysfunction, as reflected by placenta mtDNA content and/or progesterone, is related to the risk of preterm delivery

2) Pregnant women living with HIV who are treated with cART will have significantly lower placental progesterone levels and/or altered mtDNA content compared to HIV- controls.

3) Among the women living with HIV, those treated with boosted-PI cART will show lower placental progesterone levels and/or altered mtDNA content than those treated with nonboosted-PI cART.

2. Part I. Observational study in placenta and plasma samples from preterm births (PTB) and term births (TB)

2.1. Study design and participants

All the study participants were prospectively (2004-present) enrolled as part of two consecutive cohorts, the Pregnancy cohort (between 2004 and 2008) and the **C**hildren and Women: **A**nti**r**etroviral and **M**arkers of **A**ging (CARMA)-PREG cohort (2008 to present). These cohorts, funded through a Canadian Foundation for AIDS Research grant, then two consecutive CIHR team grants, had identical inclusion/exclusion criteria and similar protocols for data collection and sample processing. Both cohorts were led by the same investigators.

Participant enrollment took place at one of following medical centers: at BC Women and Children's Hospital – Oak tree clinic- in Vancouver (2004-present), (this center coordinates HIV care for all known HIV ínfected pregnant women in BC) or at the Centre Hospitalier Universitaire CHU-Sainte Justine in Montreal, QC (2008-present).

2.2. Eligibility and selection criteria

During the first phase from 2004 to 2012, all HIV+ pregnant women seen at Oak Tree Clinic were approached to participate. The HIV uninfected women of our cohort N = 60 were recruited entirely in Vancouver. During recruitment of HIV uninfectedwomen, an effort was made to approach possible participants with similar sociodemographic characteristics to the HIV infected group in order to reduce confounding factors (i.e. maternal age, smoking, alcohol consumption, and illicit drug use during pregnancy) between both groups. For the second phase

of the study, which has been ongoing from 2013, only HIV infected pregnant women were enrolled (**figure 9**).

Eligible women were approached for enrollment by research staff during a routine visit for their standard care. All the participants received a 20$ honorarium at each visit. A copy of the written consent can be found in **Appendix A and B**. Centre Hospitalier Universitaire Sainte-Justine in Montreal used a similar consent. HIV- controls were recruited via word of mouth and advertisements placed in downtown east side area of Vancouver, to promote enrolment of controls with similar sociodemographic characteristics to those of the HIV+ participants. **Inclusion criteria** were being pregnant; any age; known HIV status. For HIV+ women, being on cART or be willing to initiate cART during pregnancy. **Exclusion criteria** were being unable to provide informed written consent and/or adequately speak and understand English/French. A total of 196 women (136 HIV infected and 60 HIV uninfected) were included in this study. A flow-chart of the participant enrolment process and inclusion for this study is presented in **figure 9.**

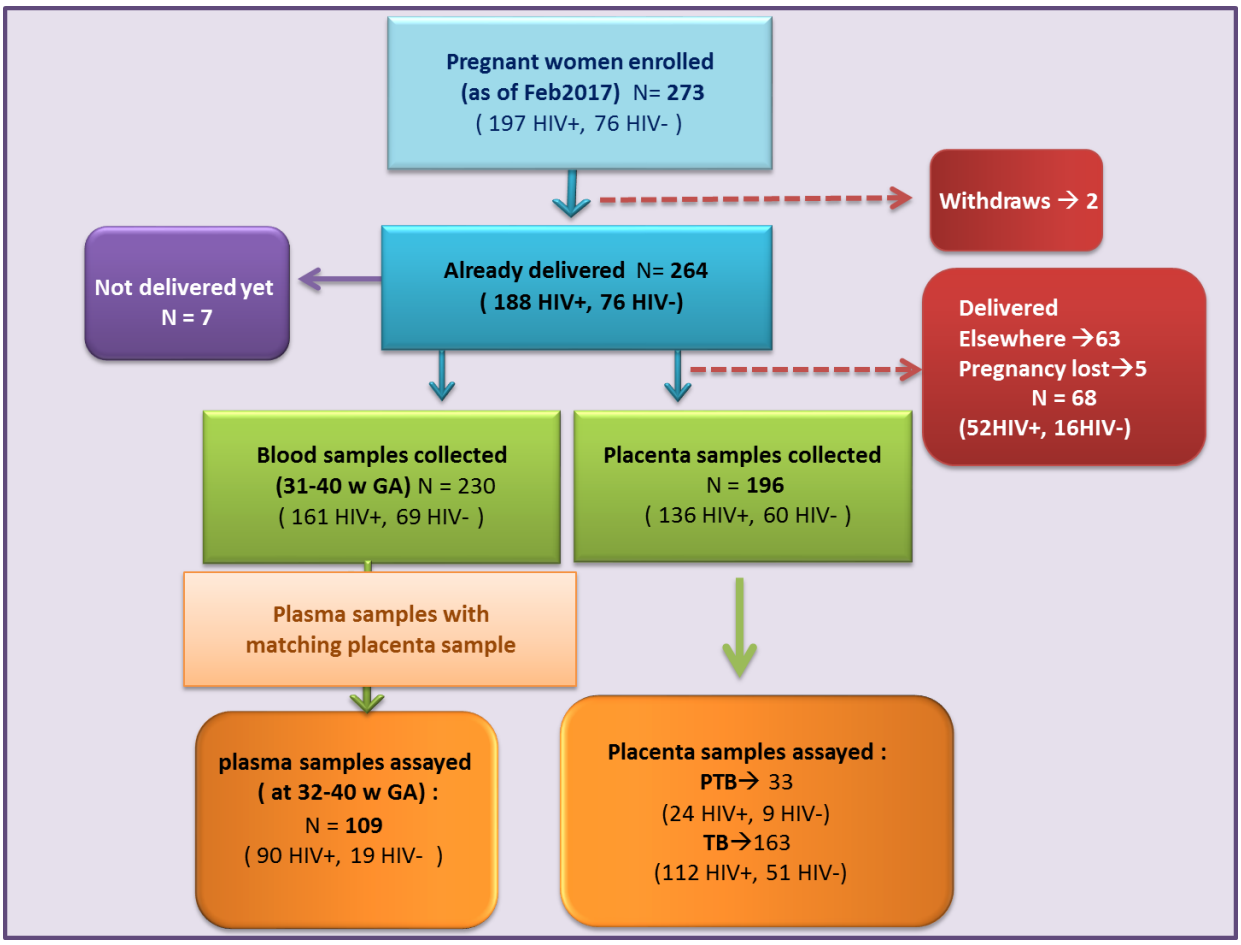

Figure 9. Selection process, exclusions, and samples assayed

There were typically three study visits during the pregnancy (13th to 23rd, 24rd to 30th and 31th to 40th weeks of gestation), one at delivery, and one 6 weeks post-*partum*. At each study visit demographic, clinical and behavioural data were collected, as well as whole blood (WB) and buccal swab samples.

The sociodemographic as well as clinical information collected included maternal age, sex, paternal and maternal dates of birth, BMI pre-pregnancy (obtained retrospectively from weight and height registered on the charts of each study participant at the moment of

enrollment), self-reported ethnicity/race, household income, and substance use history, sexual and pregnancy history among others.

The clinical data collected include details about the patients' medication history and infections. Furthermore, HIV-related parameters such as CD4 count, HIV diagnosis date as well as current and highest recorded HIV plasma viral load (pVL), ARV regimen and duration of therapy in pregnancy were also collected from the study participants.

For the substance use data (smoking, alcohol, illicit drugs), type of substance, quantity, and frequency of use was collected as text describing the type and subsequently coded for statistical purposes. For smoking, 3 codes were assigned: never smoked during pregnancy, smoked throughout pregnancy, smoke only early pregnancy-not beyond the first trimester.

Table 6 presents the sociodemographic and clinical characteristics of all the study participants. The two groups (PTB vs. term birth [TB]) were similar with respect to age, race/ethnicity and rate of infants born SGA. However, mode of delivery was different between the two groups (more C/S on the PTB group). Women, who had a premature delivery, were more likely to have had preeclampsia during pregnancy. They were also most likely to have used substances of addiction during pregnancy (alcohol, smoking or drugs) and to have delivered a baby with lower birth weight compared to those infants born at term.

Table 6. Demographic characteristics of the all the study participants (n = 196)

Characteristics		PTB (n=33)	TB (n=163)	P values
Gestational Age (weeks)		34 ± 3 [24.3 – 36.9]	39 ± 1 [37.1 – 41.1]	<0.001
Infant birth Weight (kg)		2.3 ± 0.6 [0.6 – 3.5]	3.2 ± 0.5 [2.1 – 4.4]	<0.001
Male sex		11 (33)	70 (43)	0.307
Small for Gestational Age (SGA)		4 (12)	30 (18)	0.38
Maternal HIV positive status		24 (73)	112 (69)	0.65
Maternal Age at Delivery (years)		31 ± 5 [20 - 44]	33 ± 5 [22 – 40]	0.70
Pregnancy complications	Preeclampsia	6 (18)	8 (5)	<0.001
Previous PTB		6 (18)	17 (10)	0.20
Substances of addiction during pregnancy (alcohol. smoking. drugs)		23 (70)	84 (51)	0.01
Mode of delivery	Caesarean Section	23 (70)	55 (34)	0.002
	Vaginal	10 (30)	108 (66)	
Ethnicity	Aboriginal / Indigenous	9 (27)	31 (19)	0.27
	White / Caucasian	16 (49)	63 (39)	
	Black / African Canadian	5 (15)	40 (24)	
	Asian / Others	3 (9)	29 (18)	

Table 7 presents the sociodemographic and clinical characteristics of the 136 study participants living with HIV. Among this subset of study participants, infants born preterm had a significantly lower birth weight compared to those born at term. For specific HIV parameters, such as CD4 count and viral load (closest to delivery, 3rd and 4th visits), there were no significant differences between our two groups (PTB and TB). With respect to cART regimens, both groups were similar, whereby most of the study participants in both groups (>60%) were receiving a PI-boosted regimen. **Note:** subscripts (**a**) data available for n=109, (**b**) data available for n= 125.

Table 7 Demographic characteristics of the all the participants living with HIV (n= 136)

Characteristics		PTB (n=24)	TB (n=112)	P values
Infant birth Weight (kg)		2.2 ± 0.7 [0.6 -3.5]	3.1 ± 0.4 [2 - 4.2]	<0.001
Male sex		6 (25)	46 (41)	0.14
Small for Gestational Age (SGA)		4 (17)	23 (20)	0.66
Maternal Age at Delivery (years)		32 ± 6 [20-45]	31 ± 6 [18-45]	0.52
Pregnancy complications	Preeclampsia	5 (21)	7 (6)	0.002
Previous PTB		5 (21)	14 (13)	0.28
Substance of addiction during pregnancy (alcohol. smoking. drugs) Y/N		3 (17)[a]	38 (42)[a]	0.04
Mode of delivery	Caesarean Section C/S	20 (83)	43 (38)	<0.001
	Vaginal	4 (17)	69 (62)	
cART	PI-boosted-regimen	17 (71)	72 (65)	0.5
	PI-non boosted-regimen	6 (25)	25 (22)	
	non PI-regimen	1 (4)	14 (13)	
Viral load copies/µL (closest to delivery)	Detectable (≥ 49 copies/µL)	3 (16)[a]	6(5) [b]	0.13
	Undetectable (< 49 copies/µL)	11(58) [a]	76(70)[b]	
	Unknown	5(26) [a]	27 (25) [b]	
CD4 count cells/mL³ (closest to delivery)		460 [319 - 705]	510 [340 - 690]	0.58

Table 8 presents the sociodemographic and clinical characteristics of the196 study participants dichotomized according to their HIV status. Among the HIV infected participants, 9% had preeclampsia, a rate three times higher than that observed among study participants living with HIV (9%). Both groups were fairly comparable except for the infant birth weight whereby infants born to mothers living with HIV had significant lower birth weight (p= 0.02) than those who were born to mothers without HIV. Furthermore, pregnant women living with HIV were significantly more likely to have delivered by C/S than women not living with HIV (p=0.005). **Note:** subscripts (**a**) data available for n=109, (**b**) data available for n= 125

Table 8 Demographic characteristics of the all the participants living with and without HIV (n= 196)

Characteristics		HIV infected (n= 136)	HIV uninfected (n= 60)	p value
Infant birth Weight (kg)		3.0 ± 0.6 [0.7- 4.2]	3.3 ± 0.7 [1.4 – 5.2]	**0.02**
Male sex		52 (38)	29 (48)	0.19
Small for Gestational Age (SGA)		27 (20)	7 (12)	0.16
Maternal Age at Delivery (years)		32 ± 6 [18 – 45]	32 ± 5 [21 - 43]	0.99
Pregnancy complications	Preeclampsia	12 (9)	2 (3)	0.17
Previous PTB		19 (14)	4 (7)	0.14
Substance of addiction during pregnancy (alcohol. smoking. drugs) Y/N		68 (50)	39 (65)	0.74
Mode of delivery	Caesarean Section C/S	63 (46)	15 (25)	**0.005**
	Vaginal	73 (54)	45 (75)	
cART*	PI-boosted-regimen	89 (65)	-	-
	PI-non boosted-regimen	31 (23)	-	-
	non PI-regimen	15 (11)	-	-
Viral load copies/µL (closest to delivery)	Detectable (≥ 49 copies/µL)	9 (7)[b]	-	-
	Undetectable (< 49 copies/µL)	87 (69)[b]	-	-
	Unknown	32 (26)[b]	-	-
CD4 count cells/mL^3 (closest to delivery)		500 [340 - 695]	-	-
(a) n=109 // * 1 unknown (7%)// (b) n= 125				

2.3. Methods

2.3.1. Placenta tissue

2.3.1.1. Collection and storage

Placenta tissue (chorionic villi) samples of approximately 15-20 mg were collected from both the fetal and maternal sides and at different regions (different placental cotyledons) on the organ, after removing the outer layers (amnion and chorion -on the fetal side and *decidua basalis* on the maternal side). Multiple biopsies were taken at from the superficy (~1 cm top layer) of the organ, close to either surface (maternal and fetal) from each placenta. Placenta sampling occurred usually within minutes or hours from delivery (the time since delivery was noted); samples were flash frozen in liquid nitrogen and stored at -80°C until studied. Samples from Centre Hospitalier Universitaire Sainte-Justine in Montreal, (QC) were flash frozen, then shipped on dry ice to our lab at UBC Hospital. **Note:** neither the entire placenta nor the samples collected from it were washed before or after freezing.

2.3.1.2. Total placental DNA extraction and mtDNA quantification

Total placenta DNA (nuclear as well as mitochondrial) was extracted from both maternal and fetal sides of the organ (15 to 20 mg of tissue) using QIAcube and QIAamp DNA Mini kit (Qiagen®) according to the manufacturer protocol specifications, and eluted in 100µL of buffer AE. MtDNA quantification was performed via a monochrome multiplex assay (adapted technique from Cawthon *et al* [147]) in a 96-well plate, on the LightCycler 480.

The final concentrations of reagents in the 10 µl reactions were 1X master mix (Light Cycler® SYBR Green I Master) (Roche®), 1.2mM EDTA (Sigma-Aldrich®), and 0.9 µM of each of the four qPCR primers (**table 8**), and 2 l of DNA(~20 ng/µL). All primers were purified via high-performance liquid chromatography (Integrated DNA technologies ®).

DNA extracts (40 per plate) were assayed in duplicate along with a single standard curve, a negative control and two positive internal controls (IC). The standard curve aliquots were previously prepared and the standard curve was always placed in the central column of the plate to avoid plate edge effects. It was prepared by serial dilution (1:5) of two plasmids containing Albumin and D-loop genes sequences as their region of interest, mixed 1:50. The range of the standard curve went from 253 781 250 to 81 210 copies of the D-loop gene and 5 075 625 to 1624 copies of the albumin gene. The range of linearity was 3125-fold with an R^2 >0.99. The negative control consisted of Buffer AE in place of the template DNA. The two IC (high and low mtDNA), were prepared from a 1:4 dilution of SKBR3 cell DNA (high mtDNA content control) and from pooled volunteer whole blood (low mtDNA content control).

Table 9. Forward and reverse primer sequences used to measure mtDNA content via monochrome, multiplex qPCR

Primer ID	Nucleotide sequence
Albu - F	5'- CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG -3'
Albd - R	5'- GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT -3'
D-loop_MPLX - F	5'- ACGCTCGACACACAGCACTTAAACACATCTCTGC-3'
D-loop_MPLX - R	5'-GCTCAGGTCATACAGTATGGGAGTGTGAGGGTAAAA -3'

The plates were centrifuged at 1500 x *g* for 2 min at room temperature prior mtDNA quantification. The qPCR was performed on a LightCycler® 480 using the following thermal cycling profile. Data were acquired using the LightCycler® 480 Software Version 1.5.1.62 SP. The thermal cycler setting used in our analyses are described in **table 9**.

Table 10 Thermal cycler settings for monochrome multiplex qPCR for nuclear (albumin) and mitochondrial (D-loop) genes sequences

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

MtDNA content was expressed as the ratio between the copy number of the mitochondrial gene D-loop (non-coding), and the single copy-nuclear gene albumin copy number determined via qPCR quantification. MtDNA copy number was calculated in a similar manner than the one described in a recently published paper from our lab [148].

2.3.1.3. Quality control

To assure the quality and reliability of the assay, runs were rejected and repeated if they failed to meet at least four of the following five conditions:

1) If the negative control was not negative (or was more than 3 cycles below the lowest STD).
2) If the average of all the absolutes % differences between duplicates was <10%.
3) If the difference between the efficiencies of the two genes (albumin and D-loop) was not <0.05.
4) If the single efficiency for each gene was not >1.90 but < 2.05.
5) If the mtDNA content ratio for the two IC was not within ± 2 standard deviations from the mean of all the IC ratios measured in our lab under the same conditions and using the same aliquot for STDs and IC.

Individual samples were rejected and repeated (re-assayed) if the copy number of both genes fell outside the STD curve or if the absolute % difference between duplicates was > 15%. To assess the reproducibility of our results, we randomly selected 17 samples and re-assayed them. MtDNA content values were plotted on a linear regression curve. To assess correlation, Pearson's correlation coefficient and slope of the best-fit line were calculated.

2.3.1.4. Placenta progesterone extraction and quantification

2.3.1.4.1. Tissue homogenization

For time and cost reasons, we only extracted progesterone from the fetal side of the placenta. Previously labeled 2 ml screw-cap tubes (Sarstedt ®) were weighted on an analytical balance. After recording the weight of the empty tube, approximately 10 to 30 mg of the placenta was cut from the center of the tissue and placed into a 2mL screw cap tube for re-weight.

After weighting, 400-µL of 1X PBS was added to each tube. Placenta samples were homogenized on ice using a tissue ruptor (Qiagen ®, TR 12510981) with a homogenizing probe for 15 to 20 seconds at the medium speed setting, after homogenization, 400 µL of 1X PBS was added to each tube. Homogenized samples were kept at -80°C until the moment of extraction.

2.3.1.4.2. Progesterone extraction from placenta homogenate

Homogenates were thawed at room temperature and vortexed. Diethyl ether (Sigma-Aldrich ®, cat# 296082-1L) (800 µL) was added to each homogenate which were then vortexed for 15 seconds and placed on a shaker at maximum speed for 2 min. The tubes were then left undisturbed until the liquid settled (for approximately 5 min) after which 150 µL of the top ether layer was transferred to 5 mL sterile tubes (VWR ®, Eppendorf ®). 800 µL of diethyl ether was added and the above process was repeated one more time. Once the first and second placental progesterone extracts were collected and unified in a single tube, they were kept at -80C until needed. Prior progesterone quantification, samples were removed from the freezer and let open in the fume hood in order for the diethyl ether to completely evaporate (approximately 3 hours). The extracted progesterone was then, suspended in 250µL of the assay buffer. All samples were diluted 1:10 with assay buffer before assaying them.

2.3.1.4.3. Placental progesterone quantification by ELISA

Placental progesterone levels were measured by commercial ELISA kit (Enzo lifescience ®), catalog numbers ADI-900-011 and ADI-901-011, according to the manufacturer specifications **(table 10**), using a spectrophotometer (Spectra Max m2, Molecular devices ®, serial number SMP500-01502-KNCH). Placenta extracts were diluted 1:1000 with assay buffer – provided with the ELISA kit- in order for our samples to fall within the standard curve provided by the manufacturer (6 standards, diluted 1:2 ranging from 500 to 15.62 pg progesterone /mL). Every 96 well plate contained a set of 6 standards (STD) plus 4 controls for the assay, as follows:

1) Non-specific binding (NSB) -controls for the dilution of the sample and matrix effect (how the assay buffer affects the results)-
2) Total activity (TA) - controls for the enzymatic activity. Confirms that the enzyme is working properly-
3) Blank – controls for the substrate that is added to all the wells -
4) B_o (0 pg /mL STD), controls for the maximum binding of the antibody.

The difference between the Blank and the NSB indicates how much the background of the diluent agent (assay buffer) affects our results. All progesterone values (pg/mL) obtained were normalized to the placenta weight (in grams) of each sample. Final vales of progesterone were expressed in ng/g.

Table 11. Progesterone ELISA protocol flowchart

Well ID	Blank (µL)	TA (µL)	NSB (µL)	B_o (µL)	STDs (µL)	Samples (µL)
Standard diluent	---	---	100	100	---	---
Assay buffer	---	---	50	---	---	---
STDs or samples	---	---	---	---	100	100
Conjugate	---	---	50	50	50	50
Antibody	---	---	---	50	50	50
Incubation 2h at RT (shaking)	» » »	» » »	» » »	» » »	» » »	» » »
Wash 3X 400µL	» » »	» » »	» » »	» » »	» » »	» » »
Conjugate	---	5	---	---	---	---
Substrate	200	200	200	200	200	200
Incubation 45 min at RT	» » »	» » »	» » »	» » »	» » »	» » »
Stop solution	50	50	50	50	50	50

2.3.2. Plasma samples

2.3.2.1. Collection and storage

At each visit, a blood sample was collected by arm vein puncture, into an ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer ®) or ACD tube. All the WB samples were shipped at room temperature to the BC Women's Hospital in Vancouver. Plasma samples were prepared by centrifuging 1mL of WB at 14 000 x g for 5 min, then transferring the plasma to a separate tube kept at -80°C until needed.

2.3.2.1.1. Plasma progesterone extraction

Progesterone was extracted from the plasma sample collected closest to delivery (3rd or 4th study visit, 31st to 40th week of gestation) in a similar manner to that used for placenta, described

in **section 2.3.1.4.2. Progesterone extraction from placenta homogenate,** but using 50 µl of plasma, mixed with 350 µl of 1xPBS. Plasma progesterone values are expressed in pg/mL .

2.3.2.1.2. Plasma progesterone quantification

Plasma progesterone was quantified via commercial ELISA as previously described in **section 2.3.1.4.3.** Briefly, the extract was diluted 1:100 dilution with assay buffer in order for them to fall within the range of linearity of the assay.

2.3.3. Statistical analyses and tests used

2.3.3.1. Univariate models

Data were assessed for normality via Shapiro-Wilk test. If not normally distributed, the data were log or square root transformed. Univariate associations between our measures and variables of interest were investigated by Mann-Whitney, two-tailed Student's t-test (for continuous variables, non-parametric and parametric tests, respectively), Chi-squared (for categorical variables), Kruskal Wallis multi-group comparison (with Dunn's correction to compensate for multiple comparisons if appropriate) as well as Spearman's and Pearson's correlation tests. A p-value ≤ 0.05 was considered significant for all statistical tests. All the statistical analyses were conducted in XLSTAT 2013 v 1.01 (Addinsoft ®, NY, USA) or GraphPad Prism® version 7.03.

Covariates considered in all univariate analyses included:

- Maternal age
- Infant sex
- Infant weight at birth
- SGA (yes/no)

- Maternal HIV status (HIV+ *vs.* HIV-)
- Maternal self-reported ethnicity (Indigenous, Caucasians, African Canadian and Asian/other –i.e mixed ethnicities-)
- Smoking status during pregnancy (never smoked, smoked throughout pregnancy, quit during the first trimester)
- Preterm delivery (< 37w gestation, any etiology)
- cART type (nonboosted-PI PI *vs*. boosted-PI)
- cART backbone (AZT/3TC vs. ABC/3TC vs. other) during pregnancy
- Body mass index (BMI) pre-pregnancy (underweight if BMI < 18.5 kg/m^2-, healthy weight 18.5-24.9 kg/m^2, overweight 25-29.9 kg/m^2, obese type I 30 – 34.9 kg/m^{2}and obese type II, > 35 kg/m^2)
- PE (yes/no)
- C/S (emergent, urgent, elective)

2.3.3.2. Multivariable linear regression models

Multivariate models (ANCOVA) were created using univariately important variables ($p<0.1$). For those variables that were collinear (as determined by contingency table and Chi-square test analyses), only one of them was included in the model (the one that provided the best fit (R^2) for the model).

2.4. Results from the clinical study

2.4.1. Clinical characteristics

Placental tissue was obtained from 136 HIV infected and 60 HIV uninfected participants aged 18 to 45y, of whom 24 (18%) and 9 (15%) had a preterm delivery respectively. Out of the

136 pregnant women living with HIV that were enrolled in the study, 120 (88.2%) received a PI-based regimen, of which, 96 (80%) received ritonavir-boosted-PI and 24 (20%) received a nonboosted-PI (NFV). The remaining 15 study participants (11%) received NNRTI or other (*i.e* INSTI, FI, EI)-based regimens. If a participant changed cART combo during pregnancy, the cART regimen with the longest *in utero* exposure was the one considered. For one study participant, we were unable to obtain information regarding her HIV medication.

The drugs used among the women in our study were as follows: 3TC n=116, RTV n=88, AZT n=84, LPV n=59, NFV n=33, ABC n=32, ATV n=24, TDF n=22, FTC n=16, DRV n=7, NVP n=7, RAL n=5, EFV n=3, DDI n=3, DTG n=3, d4T n=2, ETV n=1, RPV n=1. In terms of the backbone, the most common among our women was AZT+3TC, with n=82 (60.3%), ABC+3TC with n=28 (20.6 %) and TDF+FTC/3TC with n=20 (14.7%). Five other women were on different regimens than those mentioned above. None of the infants born to HIV+ mothers in this study became infected. However, HIV exposed uninfected (HEU) infants were born at an earlier gestational age (38 ± 2.5 vs. 39 ± 2.2 weeks of gestation, p=0.017), with a lower birth weight (2.9 ± 0.6 vs. 3.3 ± 0.7 kg, p=0.011) than controls.

2.4.2. Relationship between maternal-facing vs fetal-facing placental mtDNA

MtDNA content was measured in placenta (chorionic villus samples) collected from both sides of the organ. As depicted in **figure 10**, mtDNA content is highly correlated between the fetal and maternal side (Pearson's r = 0.74, p< 0.001). Based on this knowledge, we elected (partly for cost and time saving) to continue our experiments using only the fetal side of the organ, and it will be further referred as (F) for fetal.

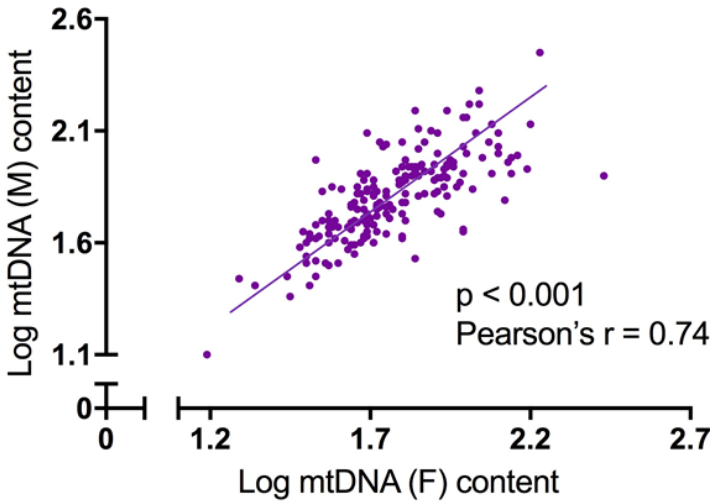

Figure 10. Pearson's correlation on placenta mtDNA, extracted from the maternal and fetal sides of the organ.

2.4.1. Relationship between progesterone and mtDNA in the placenta

Interestingly, when we explored relationships between our outcome variables we found that both, progesterone levels and mtDNA (F) content in the placenta are significantly negatively correlated. This means, higher mtDNA is associated with lower progesterone. This relationship was observed in all participants (n=196, Pearson's r = **-** 0.294, p< 0.001) **(figure 11)** and in women living with HIV (n=136, rho = **-** 0.33, p< 0.001– data not shown-) but not among the PTB group only (n=33, rho = **-**0.316, p=0.074 – data not shown-). The relationship –inverse correlation with placenta progesterone- was true also for the mtDNA (M) content (p= 0.001, rho= **-**0.235, data not shown). This increase in placental mtDNA content might be a compensatory mechanism in response to a possible placental stress or dysfunction and/or placental insufficiency. Placental insufficiency would restrict the maternal blood fluid through the placenta onto the fetus, what would translate into oxygen deficit and therefore increase of oxidative stress. Oxidative stress has been previously reported to affect mitochondria by altering mtDNA [137] [149]. Since placental mitochondrial function is necessary for progesterone

production in the placenta, this may at least partially explain the negative association that we have seen between both measures **(figure 11)**.

Another explanation for these results might be that progesterone production is associated with increased syncytiotrophoblast mass. Syncytiotrophoblast, in contrast to trophoblast, are non-dividing cells, and therefore, have lower mitochondrial activity and as such, may show lower mtDNA levels than trophoblasts, which are actively dividing cells. [125].

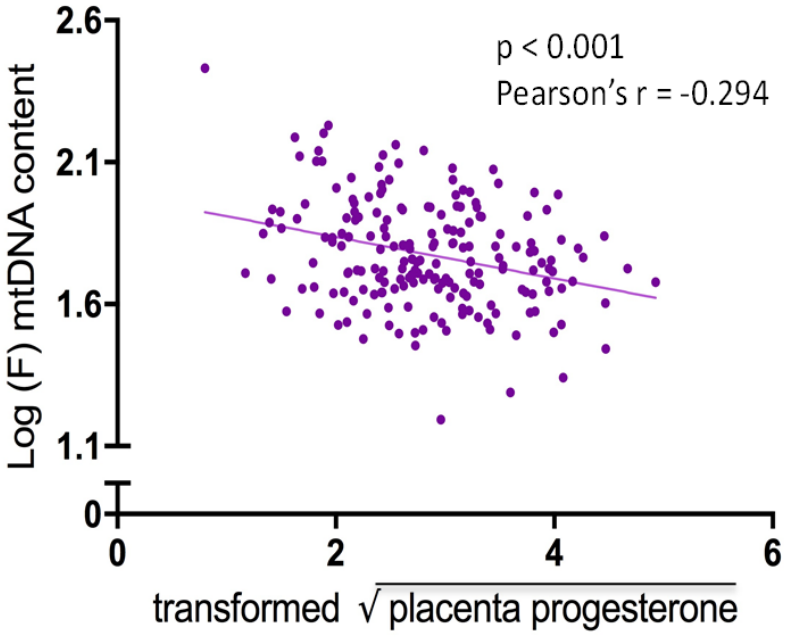

Figure 11. Pearson's correlation test between placenta progesterone –extracted from the fetal side- (square root transformed) and mtDNA content (log transformed).

2.4.2. Relationship between plasma and placenta progesterone

Although plasma progesterone is often measured, relatively little is known about how it relates to placental progesterone. To explore whether plasma progesterone levels were reflective of the progesterone levels in the placenta, we extracted progesterone from plasma obtained from our study participants at the closest visit to delivery (n=109), and related it to levels measured in

the placentae (F) (n=196). We found no association (Spearman correlation test, p=0.611, rho= 0.049) **(figure 12).**

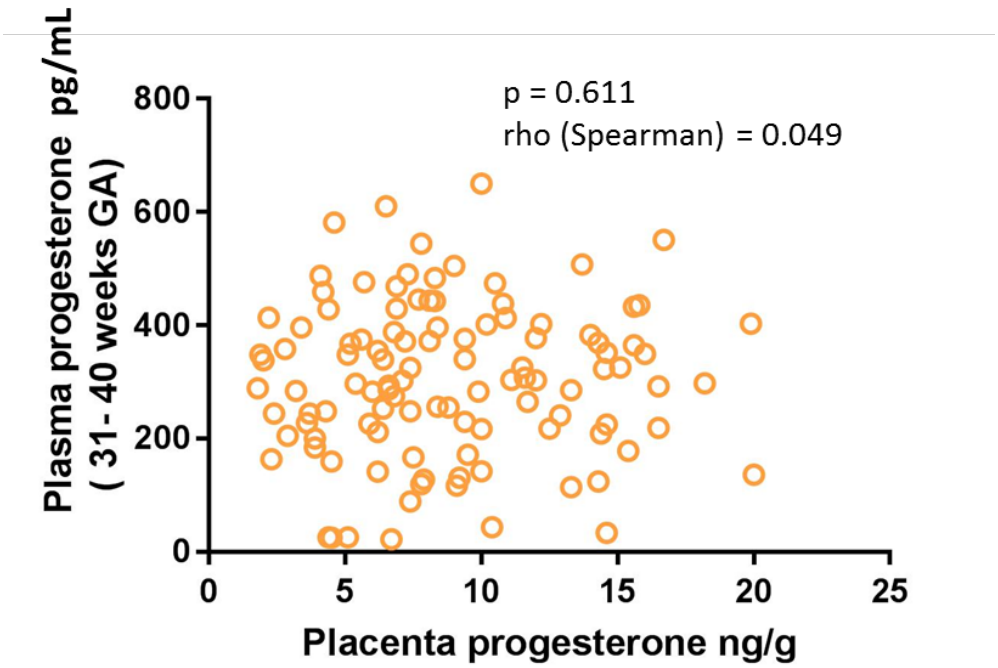

Figure 12. Spearman's correlation test between placenta and plasma progesterone levels.

2.4.3. Progesterone level and mtDNA content in relationship with HIV status

I also aimed to explore whether there were significant differences in the outcome variables (placenta progesterone and mtDNA content) in relation to our study participants' HIV status. Maternal mtDNA content was significantly higher among the HIV infected women (Mann-Whitney U test, n=195, p= 0.016, maternal mtDNA content data was missing for one participant). This difference was not seen with the placental mtDNA content on the fetal side p=0.45, **(figures 13 A and B).** This result would suggest that cART may affect mitochondria on the maternal side of the placenta more than the fetal side since the maternal blood exposure on the maternal side could perhaps be greater than on the fetal side.

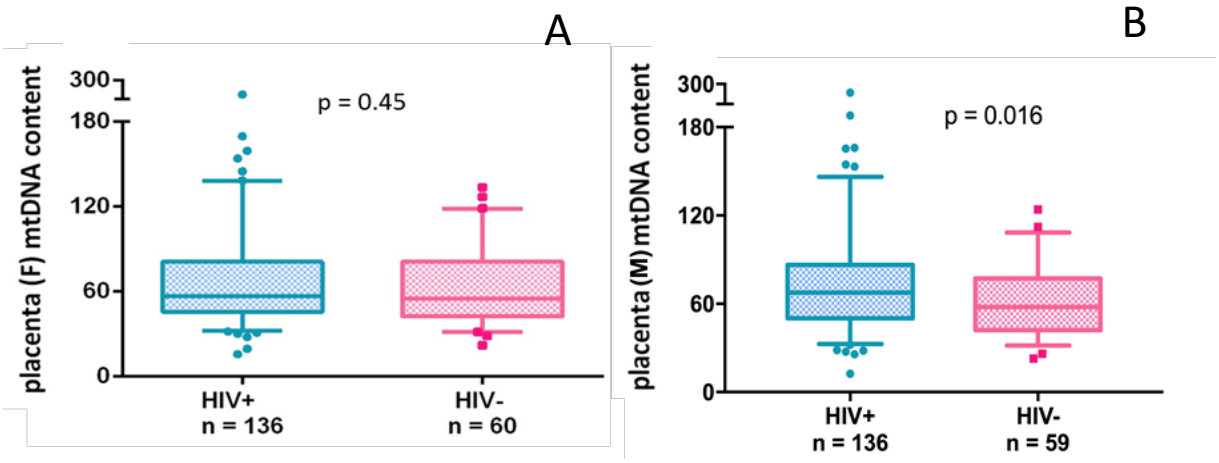

Figure13. Univariate comparisons between placenta mtDNA content from the fetal (A) as well as the maternal (B) side of the organ of HIV+ and HIV- mothers. P values from Mann-Whitney U test are shown.

After performing a two groups comparison (Mann-Whitney U test) we found that placenta progesterone level (on the fetal side) was not significantly different between the two groups, therefore not associated with HIV/cART p=0.59 **(figure 14).** Unfortunately, we did not extract nor quantify progesterone from the maternal side of the organ.

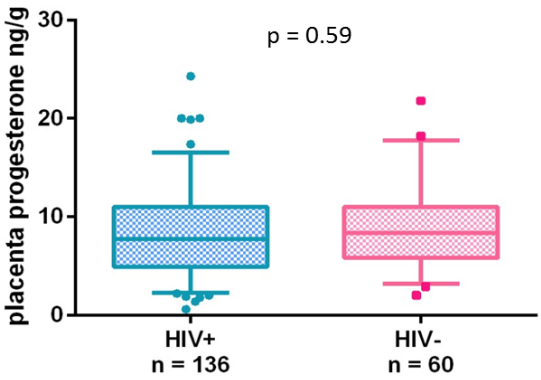

Figure 14. Univariate comparisons between placenta progesterone of HIV+ and HIV- mothers. P values from Mann-Whitney U test are shown

2.4.4. Progesterone level and mtDNA content in relationship with PTB

Within this cohort sample, PTB (all etiologies) was high in both groups (17.6% among women living with HIV and 15 % among the control women) and showed no association with HIV status, placenta mtDNA or progesterone (Mann-Whitney U test, p=0.25 for placental mtDNA content and p=0.81 for placenta progesterone levels) **(figure 15 A and B)**. The high rate of PTB observed among the HIV uninfected participants in our cohort (given that the PTB rates in BC are ~9%) might be related to the fact that HIV- participants in our cohorts shared similar sociodemographic characteristics with our HIV+ participants (poor socioeconomic status, substance use, malnutrition, multiple infections etc.) that increase the risk of preterm delivery.

We also explored if there were differences in terms of maternal mtDNA content depending on whether they had a PTB or a TB. We found no significant differences (Mann-Whitney U test, p= 0.82, n = 195 –data not shown-). In terms of the plasma progesterone, we also did not find significant differences between those women who had a PTB (n=17, 15.6%) and those who had a TB (n=92, 84.4%) Mann-Whitney U test, p= 0.947, n=109, data not shown. Our PTB group is relatively small and therefore, we may be underpowered to detect differences.

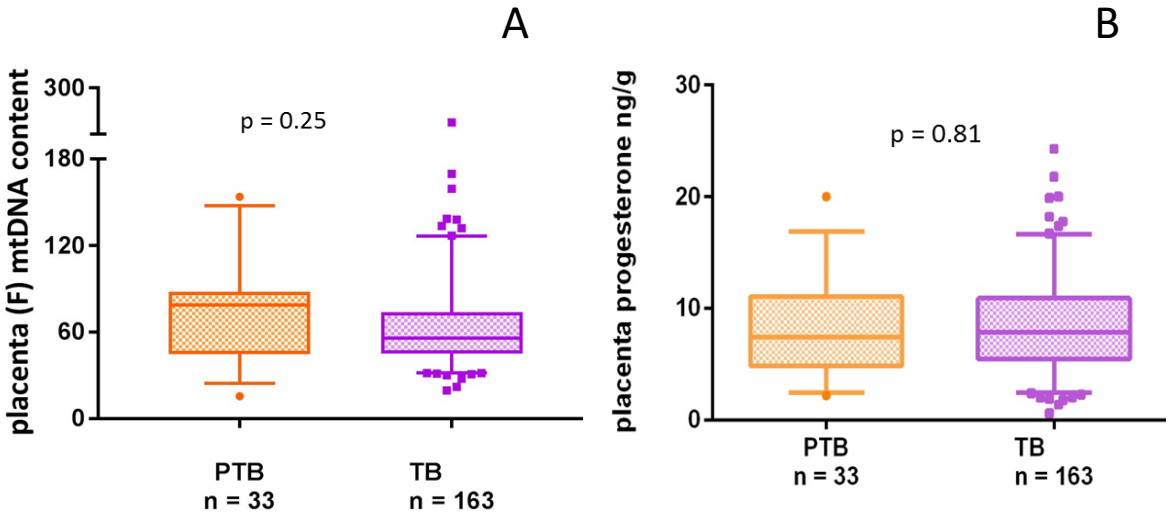

Figure 15. Univariate comparisons between placenta mtDNA content (A) as well as placenta progesterone (B) of PTB (all etiologies) and TB deliveries (all samples), independently of their HIV status. P values from Mann-Whitney U test are shown.

Given that I did not see an association between PTB with neither placenta progesterone nor mtDNA, I proceeded to explore other associations, as little is known about what factors may affect these measures. Such knowledge may inform the design of future studies, and help us address possible confounders in multivariable models.

2.4.5. Preeclampsia

When exploring whether or not there were differences in terms of placental mtDNA content among those women who had preeclampsia (previously described in **section 1.4.2.1.1**) during pregnancy, we found that this pregnancy complication was strongly associated with higher mtDNA content (Mann-Whitney U test, n=196, $p < 0.001$) **(figure 16)**.This might be explained by the fact that some cases of preeclampsia are associated with an increase in oxidative stress due to the intermittent blood flow to the placenta, what might trigger a compensatory mitochondrial biogenesis mechanism in the placenta. Proliferation of mitochondria is thought to occur as a compensatory mechanism to preserve the energy demand on the cells. Nevertheless, placenta progesterone was not associated with preeclampsia, p= 0.22 -data not shown-).

It would be interesting to further dichotomize the cases of preeclampsia according to its onset in pregnancy (early *vs* late), given the differences in terms of the risk factors, causes and outcomes associated with each pathology [150] but unfortunately that data was not available on our database. When we explored the effects of preeclampsia on the maternal side, we also found significant differences (Mann-Whitney U test, p= 0.025 -data not shown-).

Among the preeclampsia cases (n=14), 78% (n=11) had a C/S. The mode of delivery and preeclampsia were collinear according to a Chi-square test, p= 0.002, (data not shown). Also, of all the PTB cases (n=33), 18% (n=6) also had preeclampsia. Among those women who had a TB, only 5% (n=8) had preeclampsia during pregnancy. (Chi-square test, p= 0.007, data not shown). In terms of the ethnicities, out of the 14 preeclampsia cases, 50% (n=7) of them occurred among African Canadian women, 35.7% of them were among Caucasians (n=5) and the remaining were among Indigenous. The previous rates pointed that black ethnicity and preeclampsia were co-linear variables, as seen on Chi-square test, p= 0.055, data not shown.

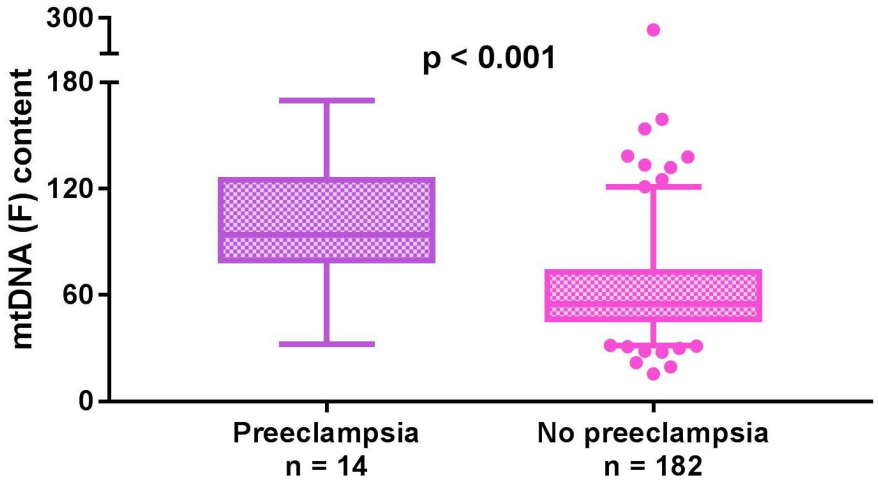

Figure 16. Univariate comparison between placenta mtDNA content of pregnancies with and without preeclampsia. P value from Mann-Whitney U test is shown.

2.4.6. Gestational diabetes

Of the 160 study participants from whom we had clinical data regarding gestational diabetes, 14% developed this condition. This rate is approximately 3 fold higher than the rate in British Columbia (which was 5.4 % in 2011 according to the Public Health Agency of Canada [151]). Exploring maternal gestational diabetes among the women in our cohort (as this might be

linked to an increase in oxidative stress [152]), we found no significant association between placental mtDNA and gestational diabetes (Mann-Whitney U test, p= 0.23 n= 160, data missing for n=36) **(figure 17)**. The same was true with respect to an association of gestational diabetes placental progesterone (Mann-Whitney U test, p= 0.47 n= 160, data not shown).

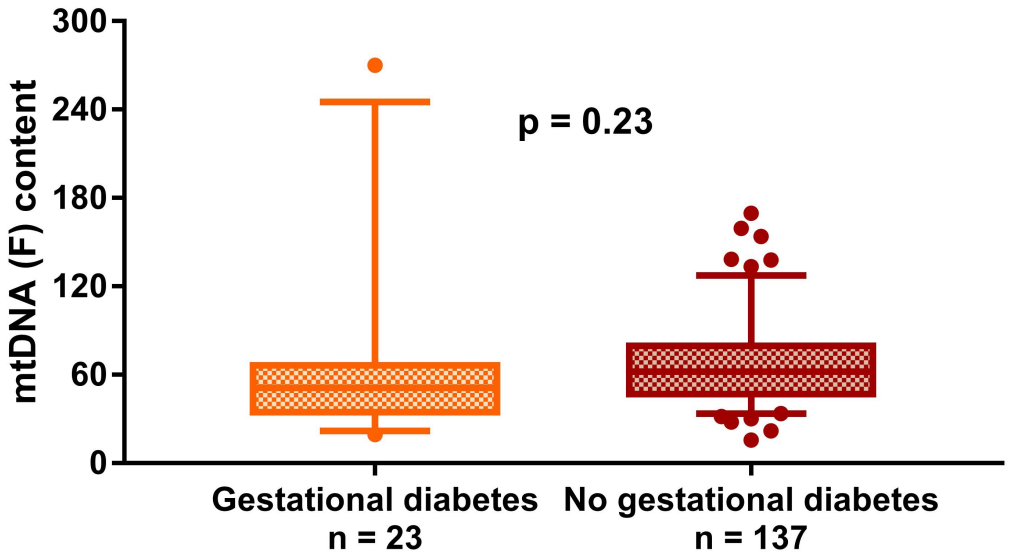

Figure 17. Univariate comparison between placental mtDNA content of pregnancies with and without gestational diabetes Mellitus. P value from Mann-Whitney U test is shown.

2.4.7. Mode of delivery

Higher placental mtDNA content was associated with having had a cesarean section (C/S) – any reason- (40%) vs. having had a vaginal delivery (60%) (Mann-Whitney U test, p= 0.037, n=196) **(figure 18)**. This could be related to the presence or absence of labour, but it could also be related to the reasons behind the need to C/S.

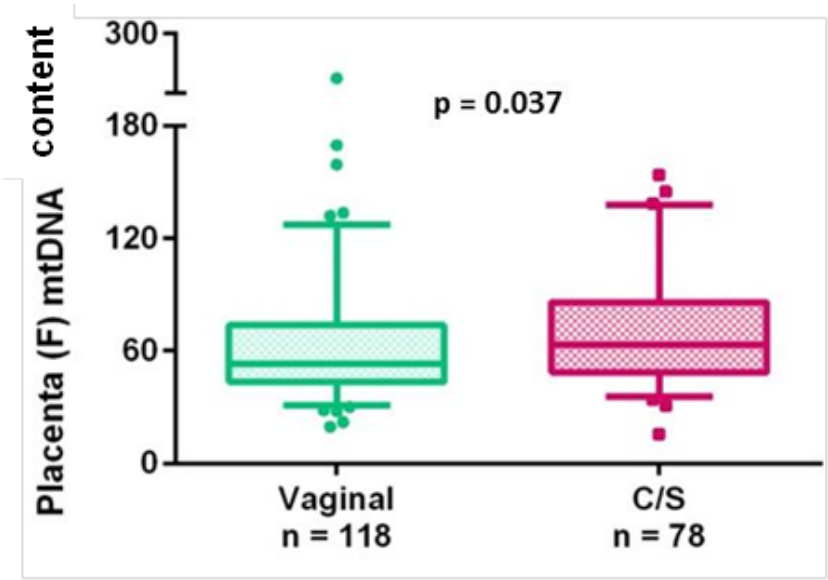

Figure 18. Univariate comparison between placental mtDNA content of pregnancies that ended via vaginal delivery or via cesarean section. P value from Mann-Whitney U test is shown.

We further trichotomized our C/S group in an attempt to discern if the etiology of the C/S had an impact on the differences observed in terms of placental mtDNA (figure 19). We observed that among the women in our study, most of the C/S (74.4%, n=58) were **emergent** (n= 47, 60.2%) or **urgent** (n=10, 12.8%) C/S, while 20 of them (25.6%) were **elective** C/S. **Note:** the etiology from one of the C/S was not available in our database.

All **elective C/S** were planned procedures [153], either prescribed by the physician (scheduled) or requested by the patient. All those **urgent C/S** (maternal or fetal compromise which is not immediately life-threatening [153]) were on short notice, but the decision was made before labour started. Finally, on the **emergent C/S** (immediate threat to life of the mother or fetus [153]**)**, the decision is made after labour had started and they were often carried out because of medical concerns about the infant's condition

In terms of mtDNA **(figure 19**) we saw **urgent** C/S having significantly higher mtNDA content than vaginal deliveries (p= 0.036) and **emergent** C/S had also higher mtDNA content than **vaginal**, but did not reach statistical significance (p= 0.063), (pairwise comparisons, Kruskal Wallis p= 0.074). This might be indicative of placenta complication (suspected on an urgent or emergent C/S). Higher placenta mtDNA appears to be a compensatory mechanism to overcome a possible placental complication or alteration. Similarly, having had a **vaginal** delivery did not show to be different than having had an **elective** C/S (p= 0.85). This might indicate that the absence (*i.e*. elective -scheduled- C/S) or presence (vaginal delivery) of labour does not affect placental mtDNA content, despite the increased energy demand seen in the placenta during labour. Similar values were seen on the maternal side.

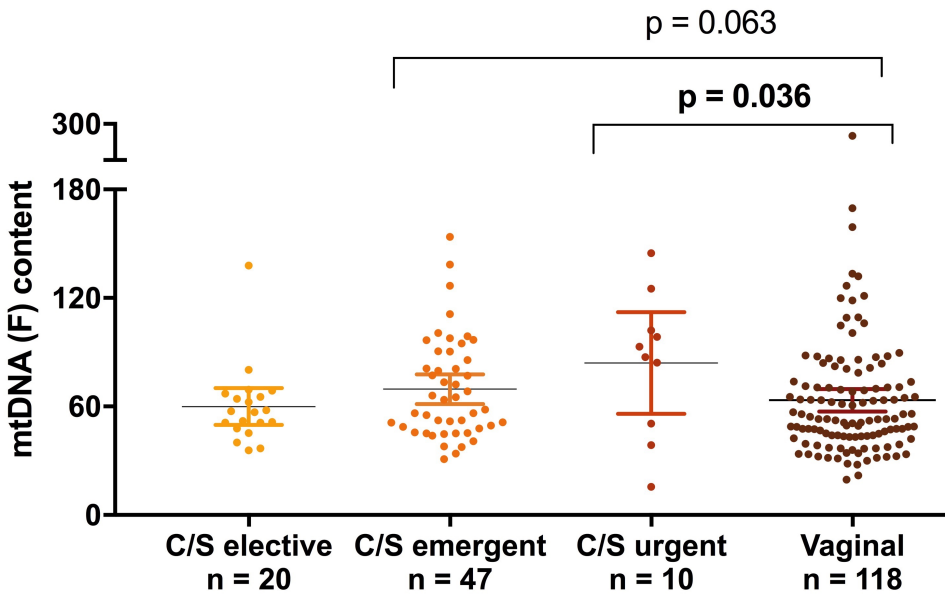

Figure 19. Univariate multi-group comparison between placental mtDNA content of pregnancies that ended via vaginal delivery or via C/Sn (elective vs emergent vs urgent). Overall p value from Kruskal Wallis test is 0.074.

2.4.8. BMI pre-pregnancy

Another risk factor for PTB is extreme BMI [80]. BMI pre-pregnancy was assessed retrospectively from each study participant's chart (height and weight at the moment of enrollment)- using the following formula: BMI= weight in kg/height in m^2. Out of the 196 pregnant women included in our study, pre-pregnancy BMI information was available for 184 women, of whom 13 (7%) were underweight prior pregnancy. The majority 100 (54%) were of healthy weight before pregnancy and 35 (19%) were overweight before starting their pregnancy, and 21 (11%) and 15 (8%) were obese type I and II, respectively. In a multi-group comparison, we found no significant associations between BMI and neither placental mtDNA or placental progesterone (via Kruskal Wallis test, n=184, data missing for n= 12, mtDNA (F) p= 0.214 and p= 0.207 for placenta progesterone) **(figure 20 A and B)**.

Note: BMI pre-pregnancy was assessed retrospectively from each study participant's chart (height and weight at the moment of enrollment)- using the following formula: BMI= weight in kg/height in m^2.

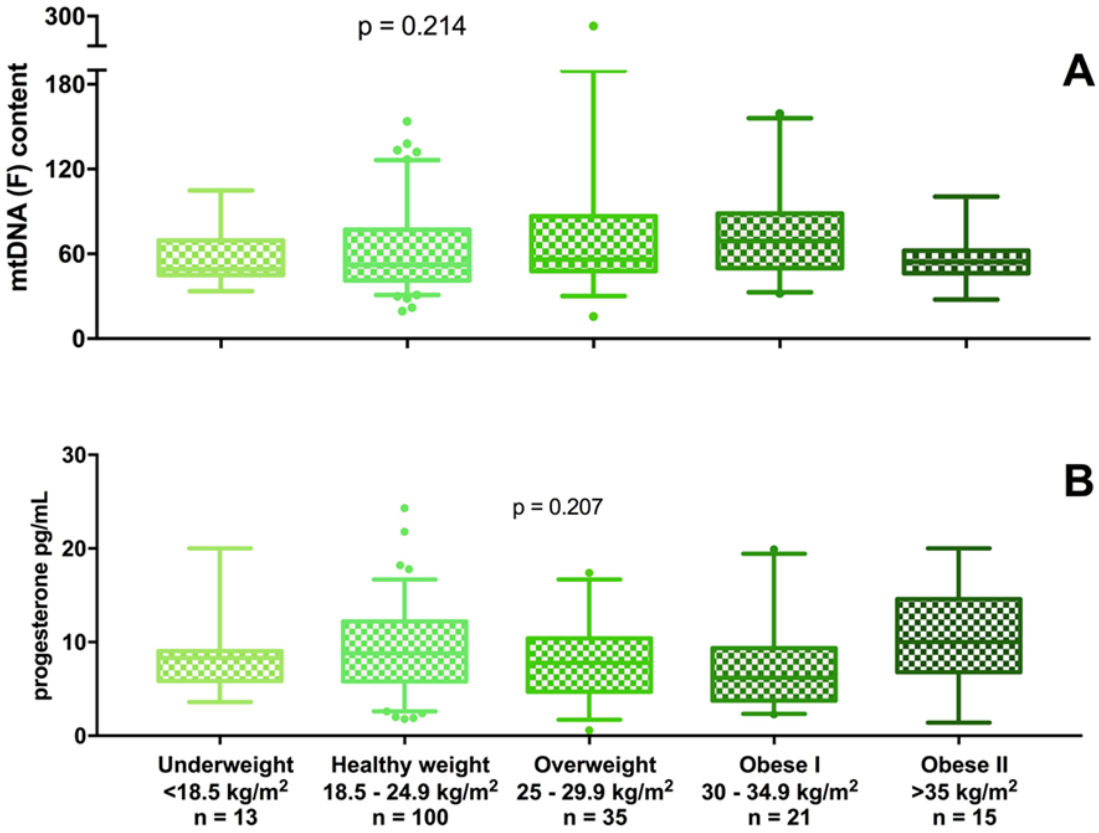

Figure 20. Univariate multi-group comparison between placenta mtDNA content (A) and progesterone (B) of pregnant women based on their BMI (kg/m^2) pre-pregnancy. P value Kruskal Wallis test.

2.4.9. Maternal ethnicity

Most study participants were Caucasians (n = 79, 40.3%), followed by African Canadians (n = 45, 23%), Indigenous (n= 40, 20.4%) and Asian or mixed ethnicity n= 32, 16.3%. There were also no significant differences in placenta progesterone based on the self-reported ethnicities of the participants (p= 0.262) **(figure 21)**.

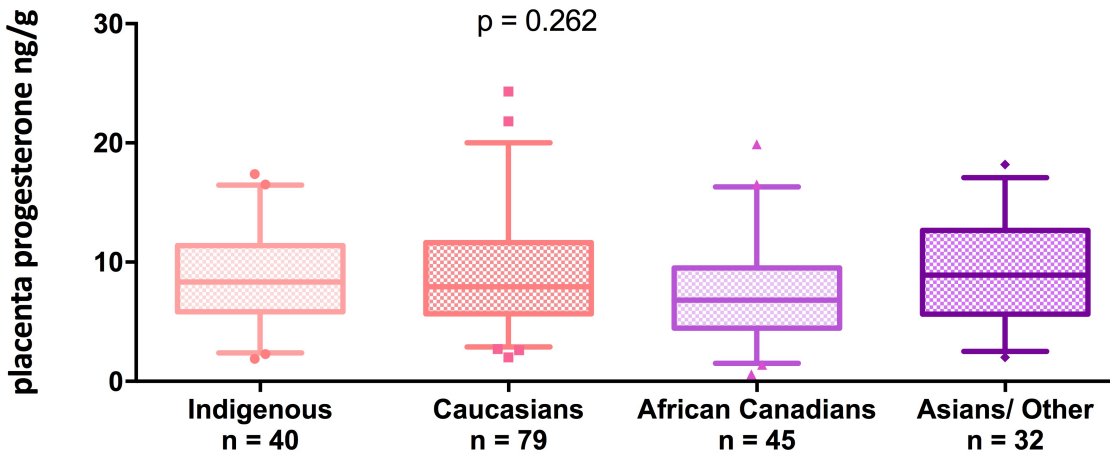

Figure 21. Univariate multi-group comparison between placenta progesterone of pregnant women based on their self-reported ethnicity. P value Kruskal Wallis test is shown

Similarly, there was no significant difference when comparing placenta mtDNA content on the fetal side between women of different ethnicities p=0.177 by Kruskal Wallis multi group comparison, n = 196 all study participants **(figure 22).**

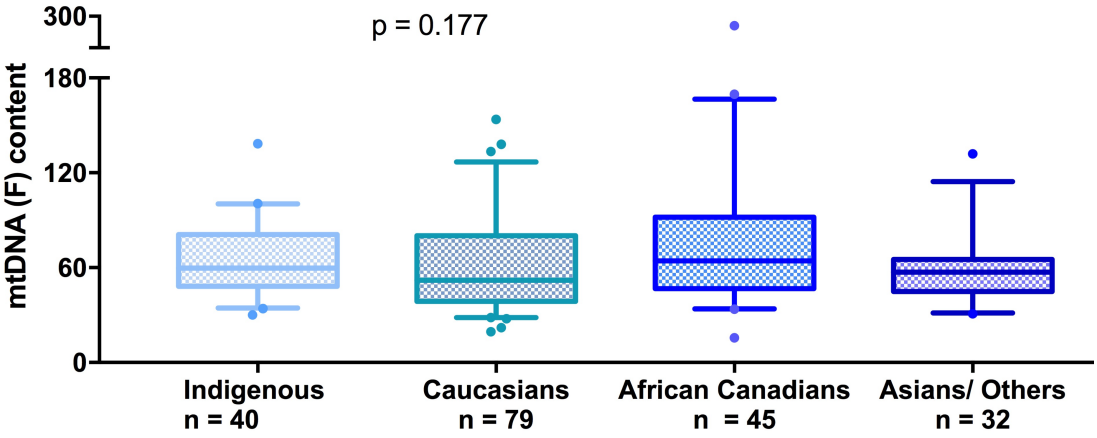

Figure 22. Univariate multi-group comparison between placenta mtDNA content obtained from the placenta`s fetal side of pregnant women based on their ethnicity. P value Kruskal Wallis test is shown

However, placenta mtDNA content on the maternal side was significantly higher among the African Canadians compared to all our groups (Kruskal-Wallis, n=195, 1 sample from the maternal side of the placenta was missing, p= 0.019). In pair-wise comparisons, mtDNA (M) were higher in African Canadians compared to Caucasians (p= 0.002) **(figure 23).**

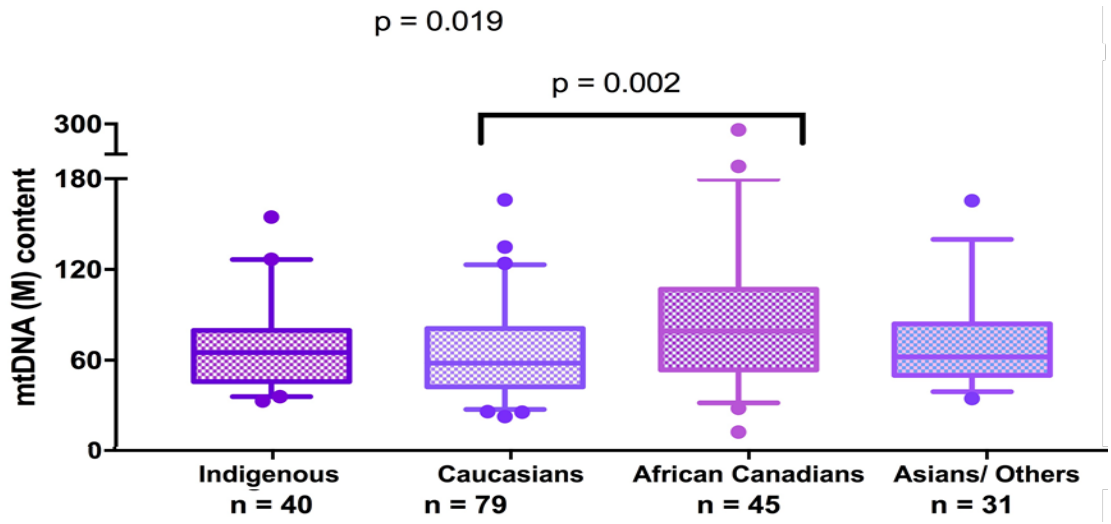

Figure 23. Univariate between-group comparison of placenta (M) mtDNA content according to self-reported ethnicity. Kruskal Wallis p value is 0.019. On the graph, the only significant pairwise comparison is shown after applying a Bonferroni's correction for multiple comparisons.

2.4.10. cART during pregnancy

In the last two decades, a growing number of studies have investigated the effects of ARVs in pregnancy. The recent literature reports that some ARVs are associated with negative pregnancy and infant outcomes [154] [33] [47] [155] and altered mtDNA content [156], specially PI-based regimens [157]. We investigated the possible association of different cART regimens with placenta progesterone and mtDNA content. Among pregnant women living with HIV who received a PI-based regimen during pregnancy (n=120), those who were on a nonboosted-PI regimen (n = 24, 20%) had significantly higher placental (M) mtDNA than those who received a ritonavir boosted-PI regimen (n = 96, 80%), p= 0.008 (pairwise comparisons). Moreover, women

who were receiving a nonboosted-PI regimen had also significantly higher mtDNA content, p<0.001 compared to the control (HIV-, no cART, n=60).

Nonetheless, no significant differences were seen between the women receiving boosted-PI regimens and those who were HIV uninfected (pairwise comparisons, p= 0.121). (Kruskal Wallis test, p= 0.002). This result may be indicative of a compensatory mechanism in placental cells to the nonboosted-PI (NFV), which is consistent with past studies suggesting that PIs affect mtDNA [116][158] [159] **(figure 24)**.

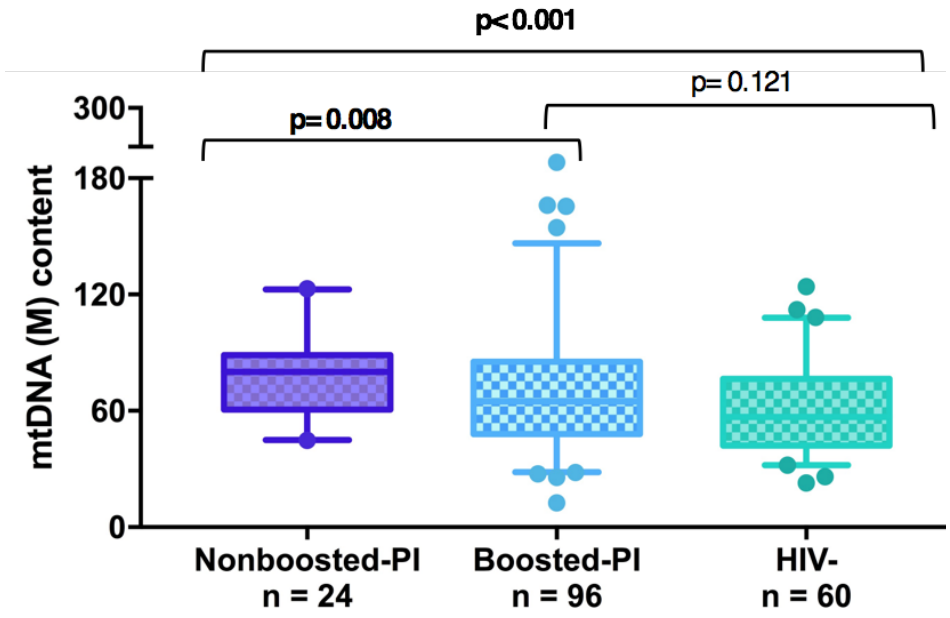

Figure 24 Univariate between-group comparison of placenta (M) mtDNA content of HIV+ women who were on PI-based regimens (boosted vs nonboosted-PI) vs HIV- (controls). Kruskal Wallis p value is 0.002, p values from pairwise comparisons shown on the graph.

No significant differences were found when we explored the effect of cART on the fetal side of the placenta, although the same trend was appreciated –nonboosted-PI tending towards having higher mtDNA levels-. (Kruskal Wallis p= 0.198 n=180, pairwise comparisons for boosted-PI vs nonboosted-PI, p= 0.11, for boosted-PI vs HIV-, p= 0.737 and for nonboosted-PI vs HIV-, p= 0.081-data not shown-). Our results suggest that both sides of the placenta are not

equally affected by cART, what is concordant with the current literature. PI are known to have a poor transfer across the placenta [64][160]. Although this might also be a consequence of some maternal tissue (i.e. *decidua basalis* or myometrium) or maternal blood contamination on the maternal side samples.

We wanted to further tricotomaze our groups based on the third drug: boosted-PI, non-boosted-PI-PI, and others –NNRTI, INSTI, etc. - (We saw significant differences only between our boosted and non-boosted (pairwise comparisons p=0.009), no differences were observed between our NNRTI and others (EI, FI) group, n =15, and the PI-based regimens (boosted and nonboosted), pairwise comparisons p=0.829 and p=0.109 respectively. Overall Kruskal Wallis test, multi-group comparison, p= 0.032, n= 135 –data not shown-).

Moreover, when we trichotomized based on the cART backbone: AZT+ 3TC, (82, 60.7%) ABC+ 3TC (28, 20.7%) and TDF+FTC/ 3TC (25, 18.6%) we found no significant differences in terms of placental mtDNA nor placental progesterone (multi-group comparison via Kruskal Wallis test, p= 0.76 and p= 0.86 respectively, n = 135, -data not shown-). Data regarding cART was missing for one of the pregnant women in our cohort.

2.4.11. Smoking during pregnancy

We also explored the effect of smoking in pregnancy, since this may result in increased oxidative stress and therefore, might cause mitochondrial damage. Among our study participants, n= 75 (38%) smoke all throughout pregnancy, n = 10 (5%) smoke only during early pregnancy – quit during the 1st trimester- and the remaining, n = 111 (56.6%) never smoke. We found no association between smoking status and placental mtDNA content (p= 0.33) **(figure 25**). There was also no relationship between smoking and placental progesterone (p= 0.41 –data not shown).

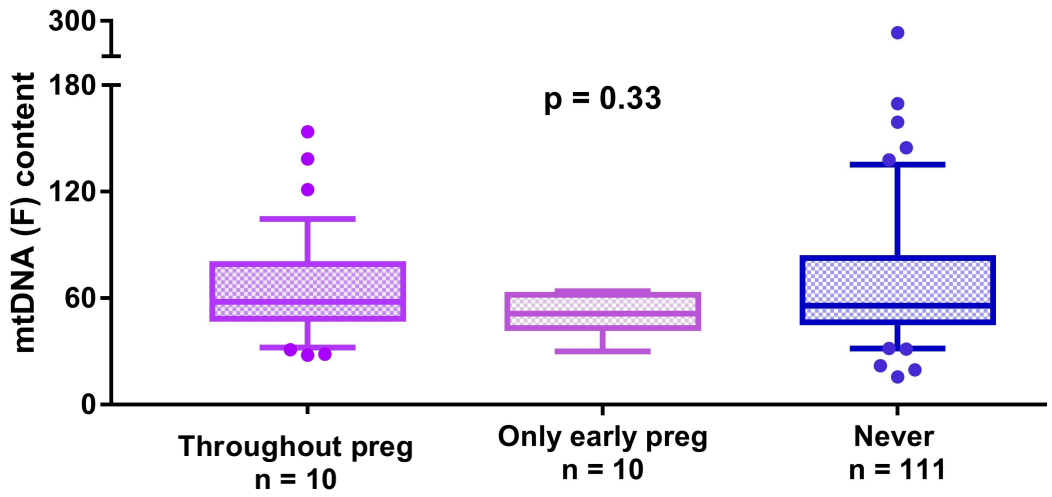

Figure 25. Univariate multi-group comparison between placenta mtDNA content of women who never smoke in pregnancy, who smoke only during early pregnancy (1st trimester) and of who smoke all throughout pregnancy. P value Kruskal Wallis test is shown

2.4.12. Multivariable models

On order to explore whether preeclampsia is independently associated with higher placenta mtDNA from the fetal side of the organ, I performed a multivariable model (ANOVA) that considered the variables that showed a significant association univariately with mtDNA. I included in my model the mode of delivery but grouped emergent and urgent C/S due to small numbers. I also included HIV status in my model since this parameter was one of my main covariate of interest. Only preeclampsia was independently associated with higher placenta mtDNA (F) content (fit of the statistical model R^2=0.097) **(figure 26)**.

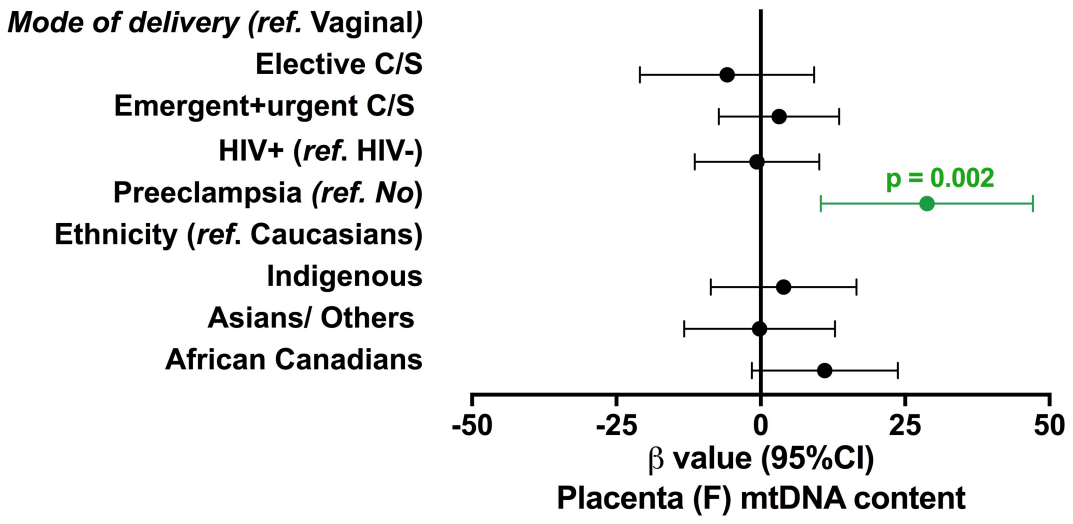

Figure 26. Multivariable model: ANOVA- Analysis of Covariance on placenta mtDNA content on the fetal side

I built the same model for the mtDNA content on the maternal side, both, being African Canadian and having had preeclampsia were co-linear variables (Chi-square test p= 0.055, 50% of the preeclampsia cases in our cohort, n=7, were seen among the African Canadian women). Only preeclampsia remained independently associated with higher placenta mtDNA (M) content. HIV was not independently associated, (fit of the statistical model R^2=0.12) **(figure 27)**.

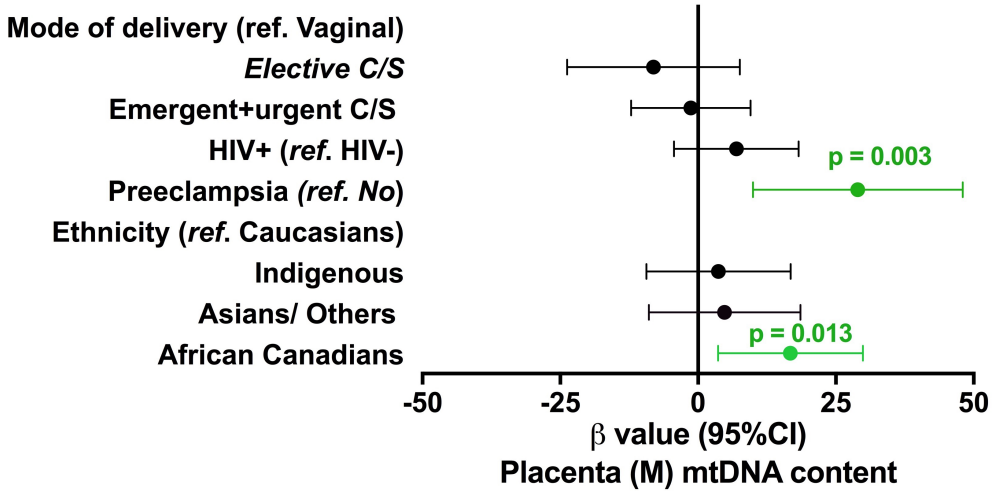

Figure 27. Multivariable model: ANOVA- Analysis of Covariance on placenta mtDNA on the maternal side

3.*In vitro* studies in two human placental and immortalized cell lines

3.1. Rationale

Currently, there is limited data on the effect of ARVs on progesterone production. The few studies that have investigated this topic were primarily focused on the possible interaction between contraceptives medications and ARVs [161] [162]. However, a recent Canadian study showed that progesterone production within a placental choriocarcinoma cell line (BeWo) was significantly reduced following exposure to 10 times the minimum effective concentration of a cART regimen used in pregnancy (AZT/3TC/LPVr -boosted -), for 24 hours [118]. They found a 25% reduction of progesterone levels in the supernatant compared to controls (BeWo cells incubated under hypoxic conditions, 1% O_2 and 99% N_2).

I aimed to further explore the effect of cART to determine whether ARVs, at pharmacologically relevant ARV concentrations, would induce alterations in the production of progesterone *in vitro*, and/or affect the mtDNA content of the cells. In our study, we used two placental cell lines, exposed them to 1X the maximum concentration achieved by the drug, during 72 hours.

3.1.1. Choice of model cell line

Two placental trophoblast cell lines, JEG-3 (American Type Culture Collection, ATCC® HTB-36™) and BeWo (ATCC® CCL-98™) were chosen. These two homo sapiens cell lines were selected because:

1. They are immortalized (choriocarcinoma), therefore they are suitable for long-term experiments.

2. They are the most commonly used cell lines in pregnancy studies, due to their derivation from the human placental trophoblast cells (outer layer of the placental chorionic villi). [163]
3. They retain their capacity to produce and secrete progesterone.
4. We have previous data from our lab showing mtDNA increase or decrease in JEG3 exposed to different ARVs.
5. Most importantly, they are biological replicates, which allows us to further validate our results.

3.2. Hypothesis and aims

Hypothesis: Progesterone production and mtDNA content will be affected in placental cell lines exposed to different cART regimens

Aim 1: To measure progesterone production and mtDNA content in placental cell lines exposed to different cART regimens at 1 C_{max}

Aim 2: To determine if there is a correlation between mtDNA content and progesterone production in our model cell lines

3.3. Methods and study design

3.3.1. Study design

We aimed to explore the effect of the cART regimens most commonly prescribed in pregnancy. **Table 11** presents the rationale for the choice of ARV studied. A total of 5 independent experiments were performed.

Table 12. cART regimens tested *in vitro* in BeWo and JEG-3 placental cell lines.

Rationale for cART tested at 1 C_{max}	Backbone	Third drug
Increasing use in pregnancy	ABC/3TC	Backbone only
		RAL
		LPV/r
		EFV
British Columbia guidelines	AZT/3TC	Backbone only
		LPV/r
		NFV
WHO guideline	TDF/FTC	Backbone only
		EFV

3.3.2. Methods

JEG-3 and BeWo cells were cultured in Eagle's Minimum Essential Medium (MEM) and F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) (GIBCO®, by life technologies ™) respectively and supplemented with 10% heat-inactivated fetal bovine serum (FBS) from GIBCO®, by life technologies ™. Both cell lines were cultured in T-75 cm^2 flasks (Falcon® ref #353131, blue vented cap, tissue culture treated) and then seeded in a 6 well-plate (Falcon®) at 20% confluence (approximately 200 000 cell/mL). Prior to seeding, cells were manually counted

using hemocytometer in a dilution of 1:10 with trypan blue. Cell viability was assessed by subtracting dead cells counting to the total cell count registered.

All ARVs were obtained from the National Institutes of Health (NIH), AIDS Reagent Program. They were dissolved in dimethyl sulfoxide (DMSO) in 10,000 C_{max} stocks (C_{max} is the highest concentration that drug reaches in plasma according to the manufacturer [164]) and stored at −20°C until used. A total of 9 different cART different regimens were prepared in a serial dilution manner as described in **Appendix D** : dilution factor 1:10, ranging from 1:10 to 1:1000) in order to achieve a final concentration of DMSO of no more than 0.1 % of the total volume per well, with a final concentration of drugs equal to 1X $C_{max.}$ For the dual –backbone only-, triple, as well as for the ritonavir-boosted regimens each drug was present on a 1 X $C_{max.}$ The 9 regimens tested are shown in **table 11.**

For the **negative control,** cells were exposed to dimethyl sulfoxide, 0.1% DMSO in cell culture media (supplemented with 10% FBS). DMSO was the dissolvent agent in which the different ARVs conditions were prepared. No antibiotic was used in any of these experiments. For the **positive control** for progesterone inhibition, we used transforming growth factor beta, TFG β (10 ng/ mL) added to the cell culture media. TGF β has been previously suggested to inhibit steroidogenesis in trophoblast cell lines [165].

3.3.2.1. Passaging, seeding, harvesting, and counting

Cells from out T75 flask stock were passaged every 3 days with 0.25% trypsin (Gibco® by Life technologies ©). During trypsinization, the flask was placed in the incubator for

approximately 5 minutes, after which, 5 mL of media (containing 10% FBS) was added. To break cell clumps we pipetted up and down several times. Once the cells clumps were broken, ~ 0.5-0.7 mL, for JEG-3, or 1-1.5 mL for BeWo was left in the flask and fresh media containing 10% FBS was added up to 18 -20mL. An aliquot of the cells (20 µL) was used for cell counting with the disposable manual hemocytometer and diluted 1:10 with trypan blue in order to find out the volume needed from the stock to seed a 6 well plate at 200,000 cells /mL per well. The desired volume was added to the wells along with 3mL of cell culture media containing 10% FBS. After 6 to 7 hours in the incubator (37°C, 5% $C0_2$), the cells were attached and the media was discarded and replaced with fresh cell culture media containing 10% FBS and the different cART regimens at 1x C_{max}.

After 72h of exposure, cell count and viability were recorded. A 1mL aliquot of the culture supernatant was collected, spun down at maximum speed for approximately one minute, and the supernatant was transferred to a new tube and kept at -20°C until needed. Once the supernatant was collected and completely removed, lysis buffer was added to each well. 200 µL of lysis buffer along with the cells lysate were collected for further DNA extraction and quantification.

3.3.2.2. mtDNA quantification

Total DNA from harvested JEG-3 and BeWo cells was extracted using the QIAamp DNA Blood Mini Kit (Qiagen) and resuspended in 200 µL Elution Buffer (Qiagen®) and stored at -20° C until needed. mtDNA content was measured by qPCR as previously described in **section 2.3.1.2**

3.3.2.3. Progesterone quantification

Progesterone from the tissue culture media (diluted 1:100 in assay buffer) was quantified via commercial ELISA, as previously described in **section 2.3.1.4.3**

3.3.2.4. Statistical analyses

Untreated cells and TGF® were included for information purpose only. This left me with 9 paired comparisons between each treatment and their respective DMSO controls. For each dataset, I performed these paired comparisons using parametrics testing one way-ANOVA, and applied a Bonferroni's correction for multiple comparisons, for all treatments showed a normal distribution according to the Shapiro-Wilks test. Graphs are presented with the mean and 95% confidence intervals. I only present the adjusted p values that remain significant after correction. **Note:** significant p values were shown on the graphs as * if $p < 0.05$, ** if $p < 0.01$ and ***if $p < 0.001$. All graphs and statistical tests were done on GraphPad Prism® version 7.03.

3.4. Results for JEG-3

Cell viability was assessed and gives us an indication of the possible toxicity of the different cART regimens. We found that the 3 backbones by themselves (ABC+3TC, AZT+3TC and TDF+FTC) affected cell viability, compared to both negative controls (untreated and 0.1% DMSO). AZT+3TC with LPVr (boosted-PI) appeared to affect cell viability the most but generally, variability was high **(figure 28)**.

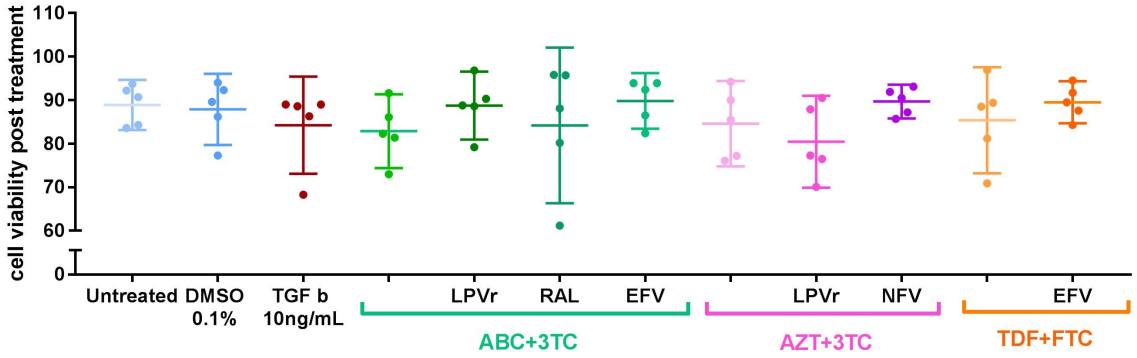

Figure 28. JEG-3 cell viability post-treatment (72h). A total of 5 independent experiments were performed. Data displayed are the means and 95% CI.

With respect to cell count after 3 days of exposure which may reflect changes in growth rate or cell death, once again, AZT+3TC+ LPVr (boosted-PI) affected cell count the most **(figure 29)**.

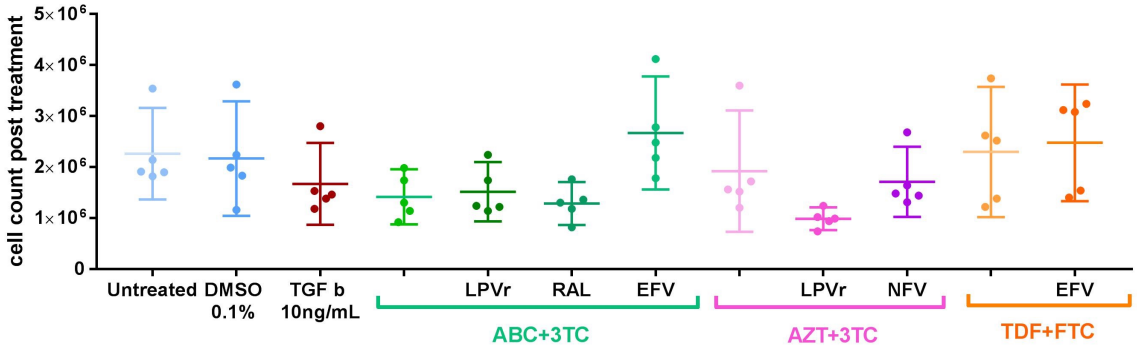

Figure 29. JEG-3 cell count post-treatment (72h), n= 5 independent experiments. Data displayed are the means and 95% CI.

When exploring the possible effect of the 9 different cART regimens on the progesterone production, we obtained unexpected results: all regimens seemed to increase progesterone production when normalized to the 0.1 % DMSO control condition although the high variability once again precludes us from reaching strong conclusions **(figure 30 B)**.

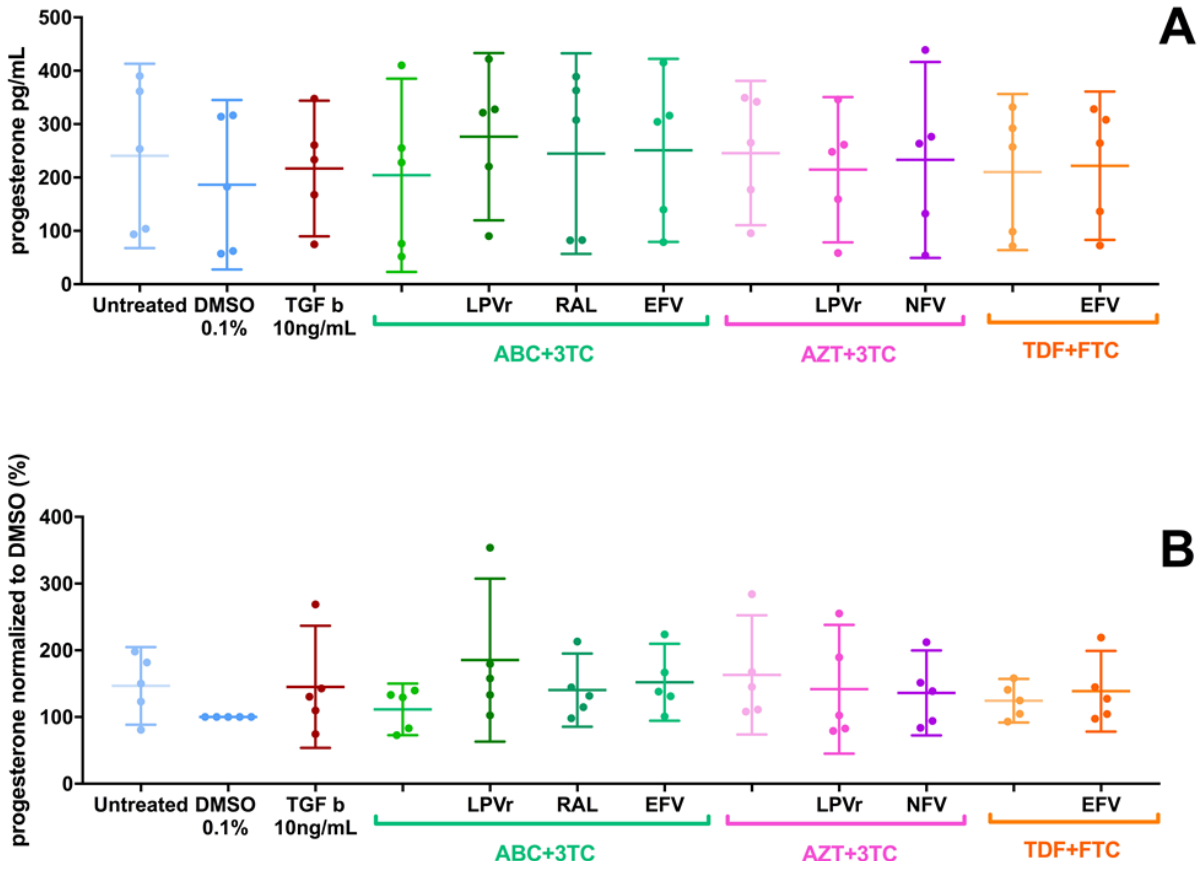

Figure 30. JEG-3 progesterone production post-treatment (72h) (A). n= 5 independent experiments. Data displayed are normalized to the DMSO (%) of each independent experiment (B), and expressed as the means and 95% CI.

When exploring the possible effects of the different cART on the mtDNA content extracted from the harvested cells post-treatment, we observed that RAL, LPVr, and NFV

seemed to decrease mtDNA content in the cells, compared to the 0.1% DMSO control **(figure 31 A and B)**.

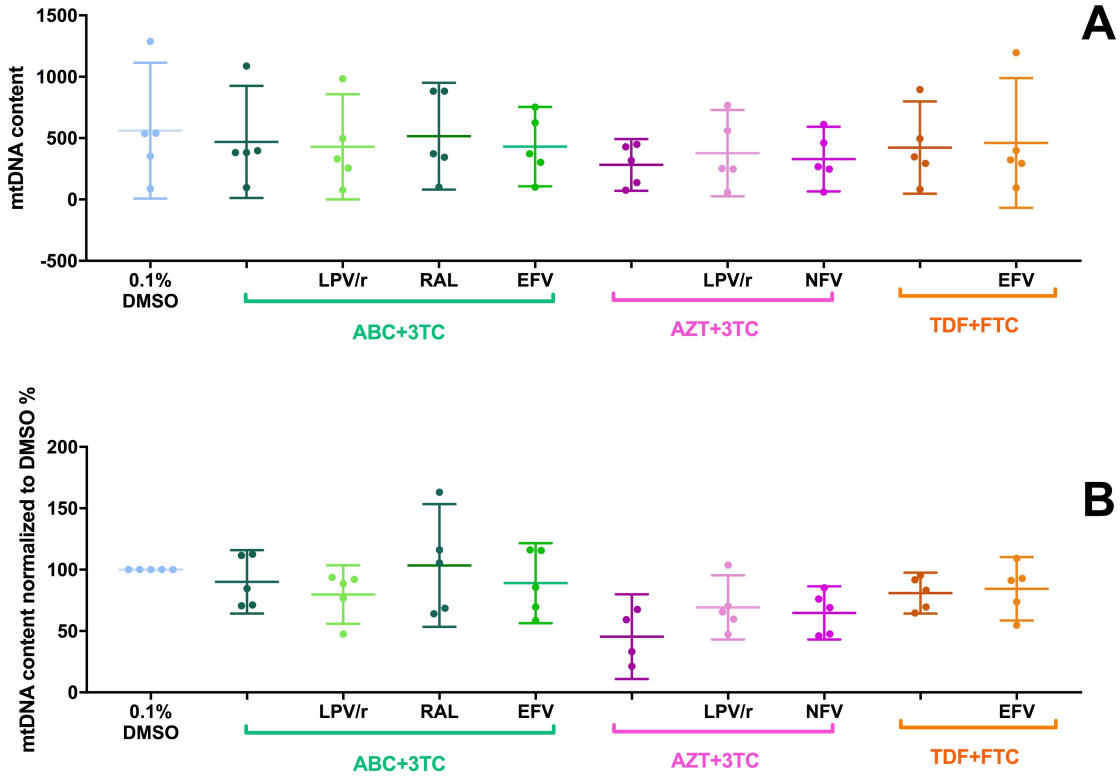

Figure 31. JEG-3 mtDNA content post-treatment (72h) (A) and normalized to the DMSO % of each independent experiment (B). A total of 5 independent experiments were performed. Data displayed are the means and 95% CI. n= 4 for TDF+FTC backbone only.

3.5. Results for BeWo

Less variability was observed for the cell viability of the BeWo replicates compared to what was seen with JEG-3 cells. Interestingly, we saw that RAL (p= 0.027) and LPVr (p= 0.002) significantly affected cell viability, followed by EFV (n.s) **(figure 32)**.

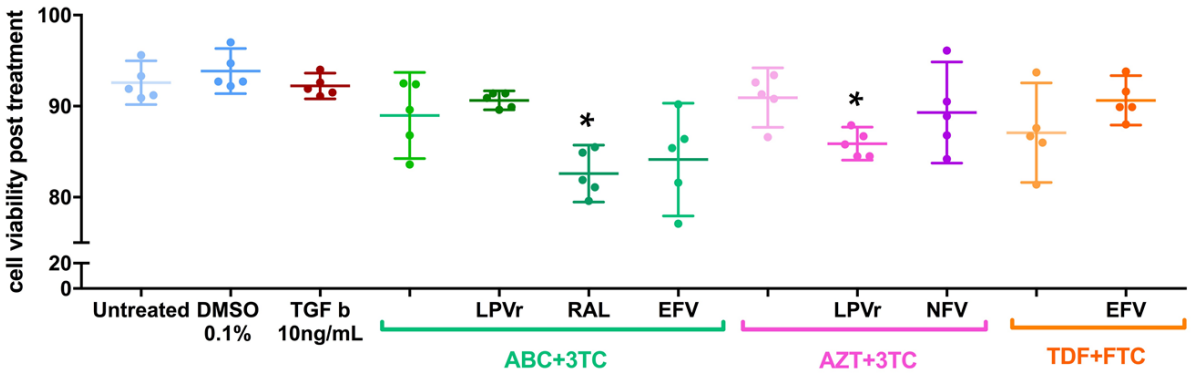

Figure 32. BeWo cell viability post-treatment (72h). A total of 5 independent experiments were performed. Data displayed are the means and 95% CI. Paired one-way ANOVA p= 0.016, adjusted p values (Bonferroni).

Upon counting the cells post-treatment, we observed that treatment with RAL (p= 0.008) and EFV (p= 0.019) yielded significantly fewer cells, either as a result of cell death, or reduced growth rate **(figure 33)**.

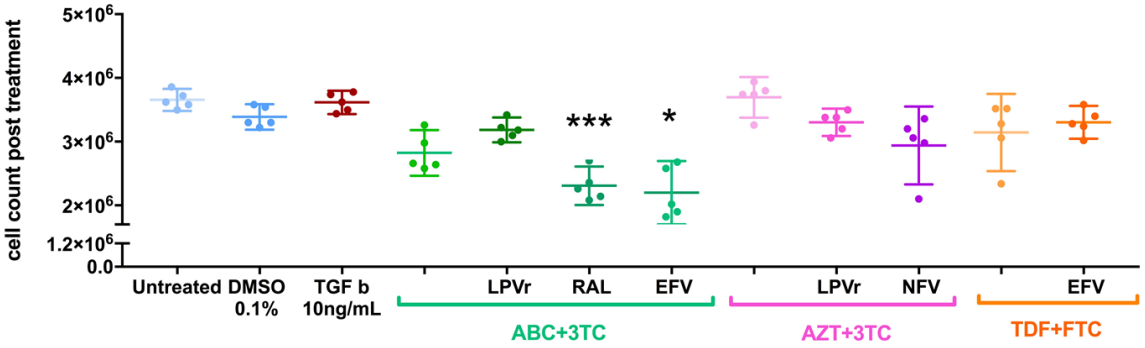

Figure 33. BeWo Cell count post-treatment (72h). A total of 5 independent experiments were performed. Data displayed are the means and 95% CI. Paired one-way ANOVA p= 0.003, adjusted p values (Bonferroni).

When assessing the level of progesterone present in the supernatant post-treatment, we found that those regimens that contained LPVr as 3rd drug seemed to increase progesterone production. Similarly, NFV and EFV seemed to increase progesterone production **(figure 34 A)**. In order to have a better sense of how the different cART regimens behaved after 3 days of exposure, we normalized to the progesterone values obtained from one of our control (0.1% DMSO, the solvent on which all the cART regimens were previously dissolved). After normalizing, NFV and LPVr yet showed higher progesterone levels present on the supernatant than the control-**(figure 34 B)**.

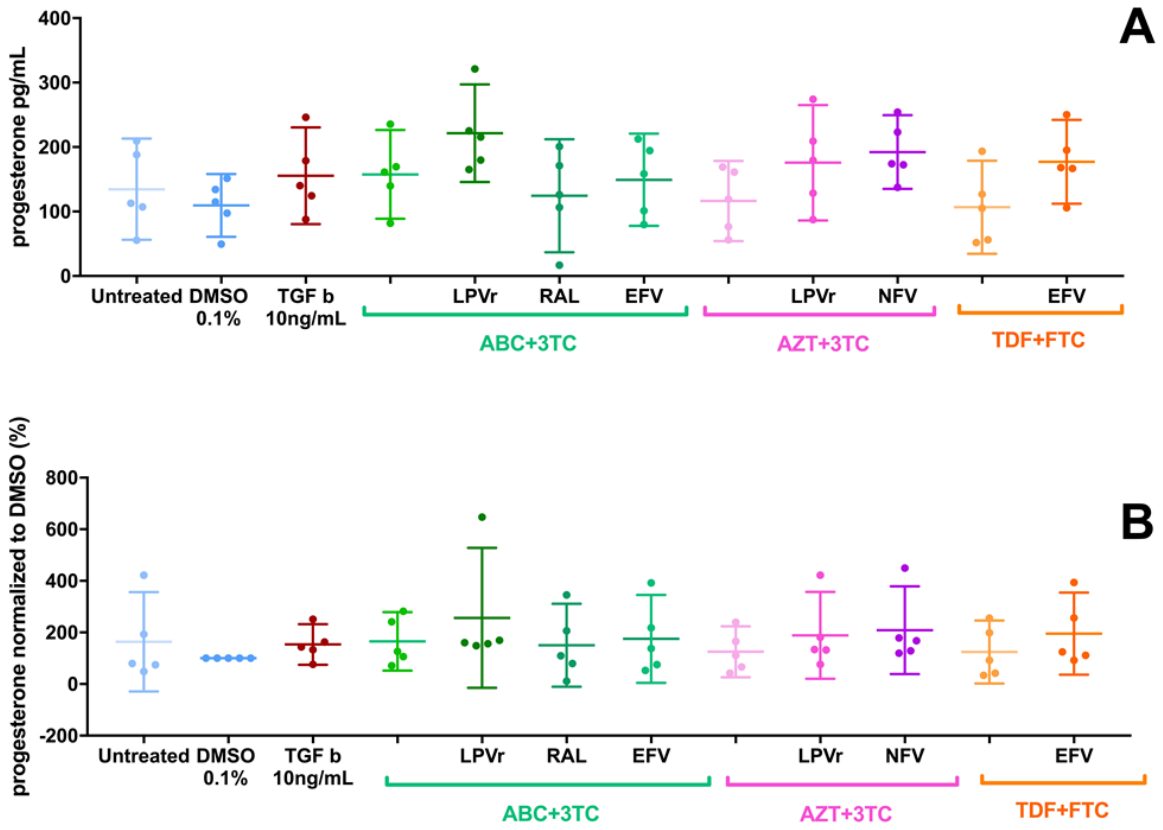

Figure 34. BeWo progesterone production post-treatment (72h) (A). Values are normalized to the value of DMSO per well (B) A total of 5 independent experiments were performed. Data displayed are the means and 95% CI

Interestingly, when exploring the possible effects of the different cART on the mtDNA content extracted from the harvested cells post-treatment, we found that LPVr (p= 0.034) and NFV (p= 0.010) significantly decreased mtDNA content, whereas EFV seemed to have the opposite effect, although not significantly, increasing mtDNA content in the cells, compared to the 0.1% DMSO control **(figure 35)**.

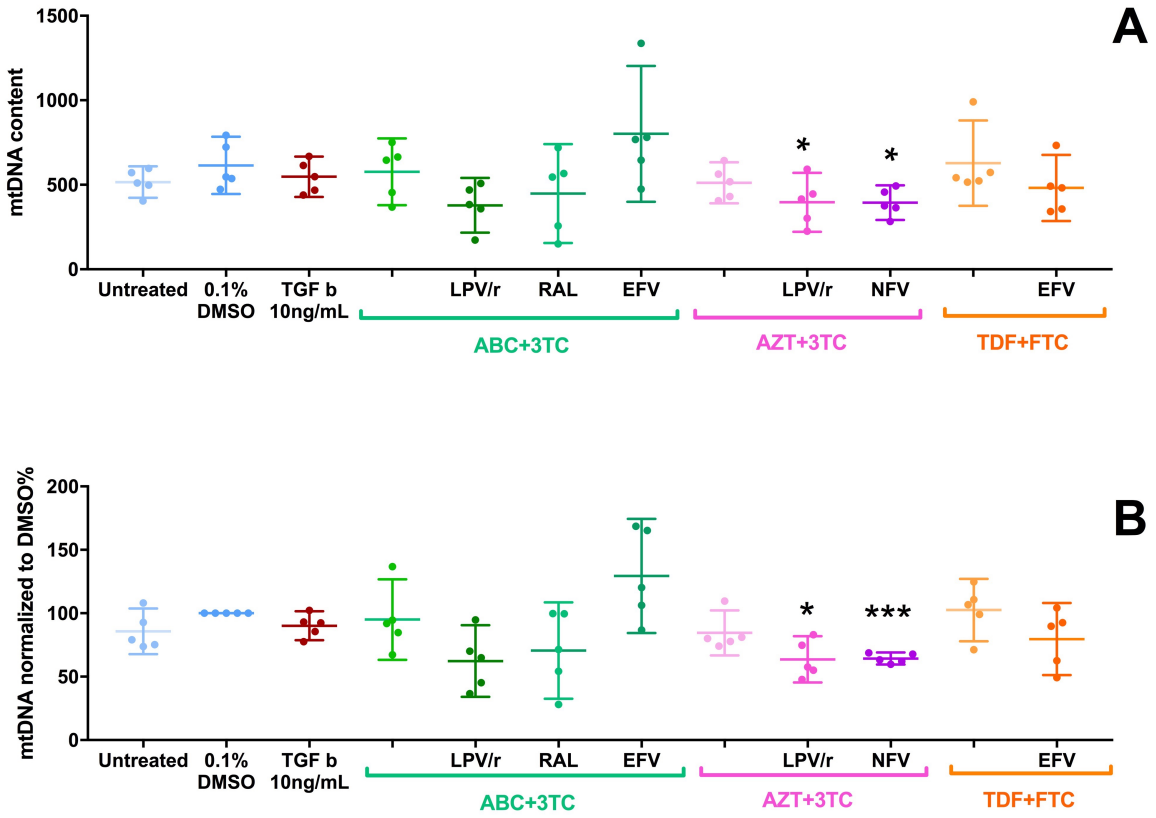

Figure 35. BeWo mtDNA content post-treatment (72h). A total of 5 independent experiments were performed. Data displayed are the means and 95% CI. Paired one-way ANOVA p= 0.0315, adjusted p values (Bonferroni).

4. Summary, limitations, and discussion

4.1. Research summary and conclusions of the clinical study

These preliminary analyses suggest a relationship between lower levels of progesterone in the placenta and increased levels of mtDNA possibly reflecting a compensatory mechanism for mitochondrial dysfunction. The latter could also explain the association we observe between preeclampsia, a negative pregnancy outcome, and elevated placental mtDNA content **(figure 36)**.

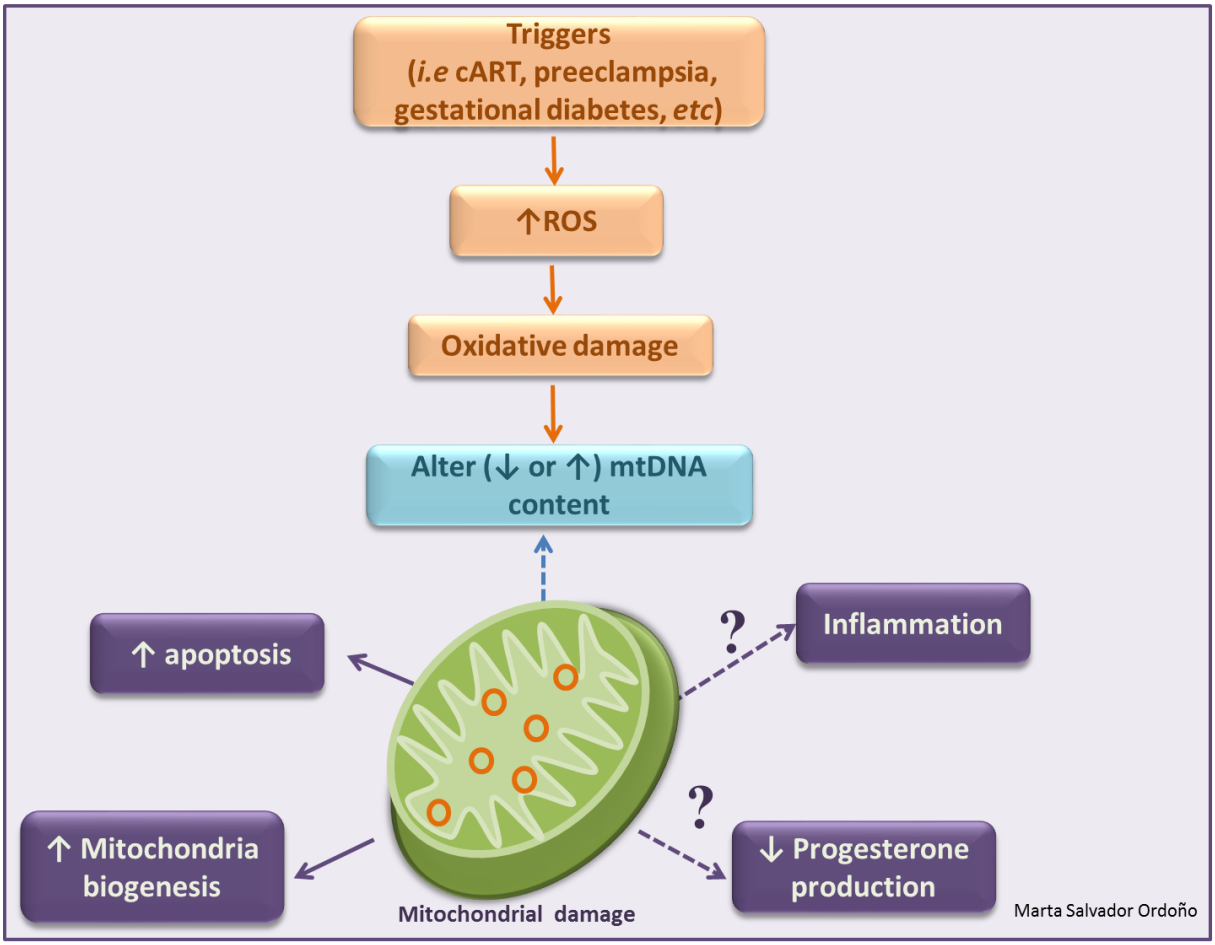

Figure 36. Theory of mitochondrial damage in response to oxidative stress

When a pregnant woman develops preeclampsia, the blood flow to the placenta is intermittent and therefore, the placenta experiences an increase in oxidative stress [166].

Consequently, during the period of reduced or interrupted blood flow (and consequent hypoxia), the placenta may compensate by increasing mitochondrial biogenesis which could explain the elevated mtDNA content we observed in the women with preeclampsia in our cohort.

In addition, higher placental mtDNA content was associated with having had a C/S –urgent-. It may be that women who had poorer placenta function (hence higher mtDNA) were more likely to deliver by C/S. Nonetheless, many other factors may be confounding this result.

Smoking during pregnancy has previously been reported to be a trigger for increased oxidative stress and therefore, might be expected to increase mitochondrial damage and impair mitochondrial function [167]. However, we saw no evidence that smoking affects placenta mtDNA.

We observed significantly higher placental mtDNA in women who received a nonboosted-PI based regimen (NFV) compared to untreated and uninfected women (HIV-). The former also had also significantly higher placenta mtDNA content than women who received a boosted-PI regimen (PI+RTV). Our results suggest an increased placental mtDNA content as a possible compensatory on the placenta cells exposed to NFV, which might indicate an increase in oxidative stress in the placental mitochondria.

In summary, some factors (i.e some ARVs – nonboosted-PIs- or preeclampsia) appear associated with increased mtDNA content, either a compensatory mechanism in response to mitochondrial damage or stress (e.g. through increased oxidative stress). The cells, in this instance, placenta cells, may compensate the insufficient energy supply by new mitochondrial

biogenesis. This could also be consistent with the association between increased mtDNA and decreased placenta progesterone. Alternatively or concurrently, decreased mitophagy could also explain the elevated levels of mtDNA.

In our analysis, both black ethnicity and preeclampsia were independently associated with higher mtDNA. It is known that black ethnicity is a risk factor for pregnant women to develop preeclampsia [78] [168]; among the participants in our study, 50% of the preeclampsia cases were African Canadians, 36% were Caucasians and the remaining were Indigenous. No cases of preeclampsia were seen among the Asians in our study.

We cannot infer causation nor draw a firm conclusion from our study but our data are consistent with previous studies suggesting increased mtDNA content as an adaptive mechanism to mitochondrial dysfunction. We proposed the working model **depicted in figure 37 although f**urther investigations are needed to elucidate the mechanism leading to higher mtDNA and whether this effectively reflects placenta insufficiency or dysfunction.

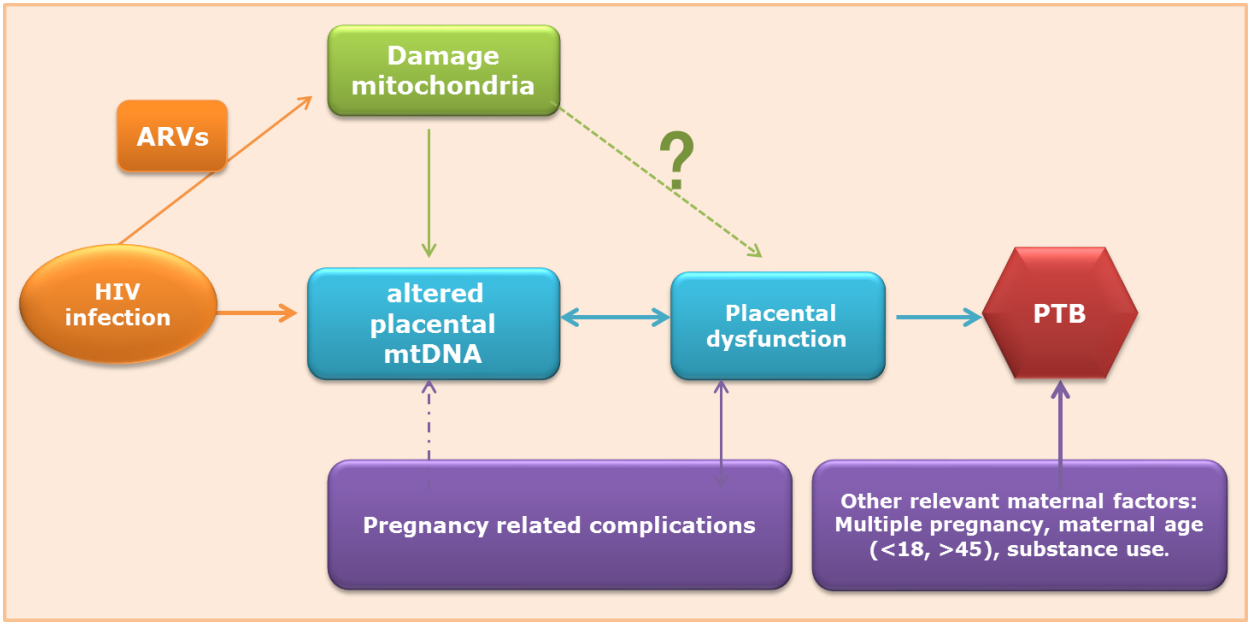

Figure 37. Proposed working model.

4.2. Research summary and conclusions of the *in vitro* study

Despite the variability observed between different experimental replicates in our *in vitro* study, we could detect similarities with respect to the effects cART exerts on our two cell lines. Cells exposed to regimens containing LPVr, RAL or NFV generally showed increased progesterone production and decreased mtDNA content. In the future, more independent experiments will be performed to compensate for the variability of this study and allow more robust conclusions regarding the possible effect of cART *in vitro*

4.3. Strengths, limitations and possible biases

Our study is the first one measuring progesterone in placenta from women living with HIV as an attempt to elucidate the possible effects of cART. Little is known about the effect of cART exposure on the placenta. Given the exploratory nature of our study, we cannot conclude on the possible mechanistic pathway of causation that may underlie the relationship between placental mtDNA content and progesterone levels. Finally, this was a sample of convenience and the study was not initially designed to answer my research question about pregnancy related complications.

Pregnancy complications were not the focus of the CARMA cohort early on. Therefore, a limitation is the study is that complete information regarding pregnancy complications was unavailable for some study participants. For example, time of onset for preeclampsia, cases of IUGR were not always reported, and the etiology for some of the PTB –spontaneous vs iatrogenic-, gestational diabetes, preterm premature rupture of membranes, *etc*.) were not

thoroughly collected for the early participants (both HIV infected and HIV uninfected) enrolled in the cohort. Another limitation of our study is that we did not have sufficient sample size to dichotomize our preeclampsia group based on ethnicity since both variables showed to be collinear.

Our HIV uninfected participants may not be representative of the general population as they are generally from poor socioeconomic circumstances and are more likely to be using substances including injection drugs. Although this is an advantage in that they resemble more closely our HIV infected participants and are in that respect a well-matched control group, it may be that our results are less translatable to the general HIV uninfected population. Furthermore, we had a small sample size for several variables (preeclampsia, PTB, etc). In terms of placenta sampling, there could be contamination of maternal tissue (*decidua basalis*) when obtaining biopsies from the maternal side of the organ.

Clinical and demographic predictors of negative pregnancy outcomes were examined. Despite being on cART and having HIV infection mostly or totally controlled, many participants likely had secondary viral and bacterial infections (hepatitis C, B, tuberculosis or chlamydia among others), other respiratory (asthma, upper respiratory tract infections, *etc.*) or pregnancy-related complications (hyperemesis *gravidarum,* bleeding, etc.), which are confounding factors that might have influenced our results but were not considered.

In terms of the recruitment of our study participants, there might be some biases among those women who were approached by the CARMA research staff. For example, only those who were able to fully communicate and provide informed consent in English were approached, therefore possibly excluding some (e.g. recent immigrants from HIV endemic countries). In addition, women who had complicated or high-risk pregnancies (i.e ectopic pregnancy, or

multiple pregnancy), at the discretion of their treating physician, may not have been approached to participate. This might mean that pregnancies with negative outcomes are under-represented in our cohort, what would be true for both groups (HIV infected and HIV uninfected).

4.4. Future directions

I would like to further explore the relationship between cART and PTB, depending on their etiology (iatrogenic *vs* spontaneous) once our sample size is larger. Unfortunately, we did not have information available for most of our participants of this important parameter. Dichotomizing PTB according to its cause would be of great importance in order to investigate if altered levels of mtDNA or progesterone have an association with those PTB occurred spontaneously and without a known cause.

I would like to explore mechanistic pathways to better understand the role of the placental mitochondria in the progesterone synthesis during pregnancy. To do so, we could also measure placenta pregnenolone, the precursor of progesterone, a relate those measurements to the progesterone previously measured in the placenta tissue from our study participants. Also, assays to measure the enzymatic activity of the cytochrome P450, responsible for the conversion of cholesterol into pregnenolone, could be carried out.

Overall, possible associations of our dependent variables (placental mtDNA content and placenta progesterone) with preterm delivery etiology and different pregnancy complications should be examined once the sample size is larger. Elucidating the possible mechanisms by which ARVs affect pregnancy outcome would contribute to the selection of a safer therapy for the management of HIV in pregnancy.

Future studies involving preeclampsia should study a larger sample and dichotomize between early and late onset preeclampsia.

4.5. Discussion

The goal of my thesis was to investigate retrospectively, the possible association of HIV/cART, progesterone production and placenta mtDNA content in pregnant women living with HIV and not. I also explored how my placental measures of interest may be associated with other pregnancy complications and outcomes.

My initial hypothesis was that pregnant women who had a PTB will have significantly lower placental progesterone levels and/or altered mtDNA content compared to those who had a TB. In this instance, I did not validate my hypothesis; I saw no significant differences in terms of placenta mtDNA among our study participants regarding PTB. No significant differences were seen in terms of placenta progesterone either. However, among those women living with HIV who are treated with cART, I did see significant differences in the placental mtDNA on those who were on PI-based regimens (nonboosted-PI) compared to HIV uninfected women. This would suggest that progesterone is not affected by cART and that the increased rate of PTB observed among women living with HIV is unlikely due to a decrease in the progesterone production.

My second hypothesis was that among the women living with HIV, those treated with boosted-PI cART will show lower placental progesterone levels and/or altered mtDNA content than those treated with nonboosted-PI cART. I partially verified my secondary hypothesis since we found that boosted-PI showed lower mtDNA than the ones treated with nonboosted-PI.

Furthermore, those women treated with nonboosted-PI showed significantly higher placental mtDNA than the HIV uninfected untreated control group. This would suggest that the nonboosted-PI regimen (NFV) affects mitochondria. Nevertheless, no significant differences in terms of placenta progesterone were seen regarding start regimens. This could indicate that placental cells were trying to compensate the effects of nonboosted-PI (NFV), which is consistent with past studies suggesting that PIs affect mtDNA [116].

Importantly, the increased placental mtDNA content seen in association with NFV-based treatment is consistent with several studies suggesting a pro-apoptotic activity of this protease inhibitor. For instance, Kumar.P *et al* (2010) [3] observed that NFV induced oxidative stress-mediated, caspase-independent apoptosis in a cell model. In addition, a recent review describing the anti-cancer activity of NFV (Koltai. T (2015) [4]) points out that the end result of all the NFV off-target effect includes decreased cell growth, decreased angiogenesis and invasion activity, both essential for placentation and early pregnancy. and increased apoptosis, which translates into reduced cell proliferation and increased cells death.

Other research groups have also studied the safety of the different cART regimens in pregnancy and the possible adverse effects in pregnancy. For example, a recently published observational study carried out by Zash *et al* (2017) [5] described and compared the safety of different cART started before conception among more than 47 000 HIV infected pregnant women in Botswana who delivered from 2014 to 2016. This large observational study reports several negative pregnancy or birth outcomes (PTB, SGA, stillbirth and neonatal death). They concluded that the regimen with a lower risk of adverse outcomes was TDF+FTC+EFV and ZDV+3TC+LPVr, was the one with the higher risk of PTB. Nevertheless, and even though they had a vast sample size and the study participants shared similar socio-demographic

characteristics, the observational character of this study made it difficult to account for confounding factors. In addition, NFV was not used in Botswana.

The purpose of my *in vitro* study was to explore whether or not cART at pharmacologically relevant concentration, affects progesterone production and mtDNA content in cultured placental cells, under controlled conditions, without the presence of confounders seen in human studies. Unfortunately, overall, the variability of my *in vitro* data means that very few significant differences were observed. This variability also precludes me from drawing strong conclusions and warrants either a larger sample size or yet unidentified changes in methodology to reduce variability. However, based on the patterns I saw, certain regimens (RAL as well as EFV in combination with ABC+3TC) significantly reduced the cell count in BeWo, what could indicate that these drugs are increasing cell death or slowing down cell division. Also in BeWo, we observed significantly reduced cell viability of the regimen containing RAL, as well as LPVr (in combination with AZT+3TC), this also could suggest that these drugs might increase cell death or reduce cell growth rate. Furthermore, AZT+3TC in combination with boosted (LPVr) as well as nonboosted-PI (NFV) showed a significant decrease in terms of mtDNA content compared to the DMSO control, what might suggest that boosted or not, PIs affect mtDNA, what is concordant with existing literature [158] [159]. Overall, my *in vitro* data would inform about how these experiments can be redone in the future.

Another Canadian study, carried out by Papp *et al.* (2014) showed decreased progesterone production *in vitro* (BeWo) associated with PIs (RTV, LPV, ATV) treatment. No effect was seen with NRTIs (ZDV, 3TC) or with NNRTI (NVP). On that study, cells were exposed for 24h to 10 X the minimal effective concentration of each drug and even though they

claimed that drug concentration did not affect cell proliferation, data regarding cell viability post-treatment was not reported.

Overall, I observed an association between higher placenta mtDNA and preeclampsia as well as with having an urgent C/S. This may suggest that placenta mitochondrial stress plays a role in these pregnancy outcomes, and I propose that elevated placenta mtDNA content could indicate a physiological response to mitochondrial dysfunction. However, given that this part of my study was exploratory, this observation should be replicated in an independent cohort, with a larger sample size. In addition, this association does not establish whether this mtDNA change is a consequence or a contributor to these outcomes. If my results were confirmed, this could further our understanding of preeclampsia and perhaps open research avenues for its prediction and prevention. A growing body of literature also points towards a relationship between **placental insufficiency** and **mitochondrial dysfunction** (with increase oxidative stress, a decrease of ATP production), impaired calcium homeostasis and **chronic inflammation** as the mechanism involved in some of the pregnancy-related disorders (*i.e.* preeclampsia, gestational diabetes). Consequently, the significant increase of mtDNA seen in the preeclampsia cases of my study is consistent with the current literature. For instance, a recent paper by Vishnyakova *et al.* (2016) [6] also found a significant increase in placenta mtDNA content on cases of early-onset preeclampsia (n=13) compared to controls (n= 14). This increase was not seen in the late-onset cases of preeclampsia (n= 11), which might suggest that the mitochondrial response is dependent on the physiopathology of this pregnancy-related disorder. Unfortunately, I could not dichotomize the preeclampsia cases of my study based on their onset. As the cohort was not initially designed to examine factors relevant to PTB obtaining information retroactively would be difficult logistically and it would introduce biases.

Muralimanoharan *et al* (2012) [7] reported an increase in reactive oxidative species and decrease in several complexes (I and III) of the mitochondrial ETC seen in cultured primary trophoblasts from preeclamptic placentas (n=6) compared to controls (n=6). They also observed, via electron microscopy, morphological damage in mitochondria (swollen and broken cristae) from preeclamptic placentae compared to controls (intact mitochondria). Taken together, this would also suggest that preeclampsia leads to placental mitochondrial dysfunction.

Furthermore, Hache *et al* (2011) [8] also reported impaired calcium homeostasis (what would induce cellular apoptosis), increased oxidative stress and lack of ATP in preeclamptic placentas (n=8) compared to controls (n=16). Another group (H.C. Lee *et al*. (2000) [9] reported increased mitochondrial mass and mtDNA content on cell exposed to treatment with an exogenous oxidant agent what suggest that hypoxic stress (*i.e.* due to an intermittent and insufficient blood flow seen in the preeclampsic placentas) could lead to mitochondrial biogenesis and therefore, would increase mtDNA content. One thing these studies have in common is that they are all done on a very small sample size, and therefore not adjusted for possible confounders. Much remains to be elucidated with respect to this serious pregnancy complication.

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Appendices

A.: CARMA-1 (2011-2013) & CARMA-PREG (2013-presentt) consent form

Mechanism of Aging Following Exposure to HIV Antiretroviral Drugs

CIHR Team Grant in HIV Therapy and Aging

CARMA-1: MITOCHONDRIAL AND TELOMERE STUDIES IN PREGNANCY

AND

Placental Mitochondrial Toxicity of HIV Therapy during Pregnancy:

Clinical Tool Development and Determination of Outcome Variables for Clinical Trials

AND

MEASURING MITOCHONDRIAL AGING, APPLICATION TO HIV INFECTION AND THERAPY,
AND
CELLULAR AGING AND HIV COMORBIDITIES IN WOMEN AND CHILDREN

carma-1-preg

~ Informed Consent ~ Study Participants ~

Site Principal Investigator: Dr [redacted]

[redacted]

[redacted]

Local CIHR Team Co-Investigators

[redacted]	[redacted]	[redacted]	[redacted]

	[redacted]	[redacted]	

Emergency contact 24 hrs/7 days/week ~ [redacted]

INTRODUCTION

Throughout this consent form, when we say "you" or "your", we mean you or your child. You are being invited to participate in the research study, named above, because you are taking, or will be taking, anti-HIV drugs in order to reduce transmission of HIV-infection to your baby. The study team, listed above, is trying to better understand the effects of the drugs that you are taking on the cells of your body. This team also hopes to better understand contributing factors for preterm birth in women living with HIV.

This consent form will provide you with information on the purpose of the study, how it may help you, any risks to you during pregnancy, and what is expected of you during the study. Once you understand the study, if you agree to take part in the study, you will be asked to sign this consent form. You will be given a copy of this form to keep for your records. This project is funded by a grant from the Canadian Institute of Health Research (CIHR).

PURPOSE

As you know, anti-HIV drugs reduce the chance of HIV transmission from mother to child from 25% to less than 1%. Although you are taking some of the best and safest available anti-HIV drugs to prevent virus transmission to your baby, it has been shown that some anti-HIV drugs, may have a toxic effect on the cells of the body. We suspect these effects may be responsible for changes in your blood tests resulting in abnormal liver function tests and changes in blood count (e.g. decreased hemoglobin levels). In the longer term, mitochondrial (energy producing part of body cells) toxicity (mitochondria are not working properly) has been associated with changes in blood lipids (fats in your blood) and thinning of the bones (osteoporosis) that is seen at a younger age in HIV positive persons. Some of the medications can have an effect on different body systems that leads to mitochondrial dysfunction. When the mitochondria become toxic the body can start to build up high levels of lactate (a byproduct of cellular function). Some doctors have expressed concerns that mitochondrial toxicity may be even more common in pregnant women than in other adults on these medications. We have not had good tests to assess this in usual practice. Also, when mitochondria are affected, they make molecules (small particles called free radicals) that can cause damage to DNA.

Research has shown that pregnant women living with HIV are twice as likely to have a preterm (early) birth compared to a pregnant woman without HIV. Because preterm birth is a complicated condition with several possible contributing factors, it is unclear what, if any, role is played by HIV and HIV medications as well as disruptions in the normal 'healthy' bacterial environment of the vagina.

The purpose of this study is to investigate the effect of taking anti-HIV drugs on women during pregnancy and on their infants, using two experimental laboratory tests. One is for mitochondrial DNA (mtDNA) and will test the level of function of the mitochondria and the other test will look at damage drugs may do to the length of DNA at the end of chromosomes. Additionally, we would like to better understand the relationship between preterm birth, HIV, HIV medications, the bacterial environment of the vagina and other risk factors for preterm birth.

STUDY ELIGIBILITY/SCREENING

In order to be eligible to participate, you must:

1. Be a pregnant woman of any age.
2. Have a confirmed diagnosis of HIV infection.
3. Be taking, or be going to take anti-HIV drugs during your pregnancy consisting of at least three medications from two specific classes of drugs – nucleoside reverse transcriptase inhibitor (NRTI) and either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI).
4. Have a detailed medical and medication history done.
5. Have blood drawn from a vein for routine laboratory monitoring?

You are **not eligible** to participate if you:

1. Are not HIV infected.
2. Are not pregnant.

STUDY ENTRY

If you decide to take part in this study, and you sign this consent form, the

following outline describes the study schedule.

Schedule of Visits

All of the study visits will be linked to routine clinic visits for your pregnancy and the routine follow-up of your baby. You will have 3 study visits during pregnancy, generally between 16-20 weeks gestation (or prior to starting anti-HIV drugs if you were not taking them before becoming pregnant), and at 24-28 weeks and 32-36 weeks. Another study visit will be scheduled during the time of delivery as well as at 4-8 weeks after delivery. Blood samples will be drawn at these same visit times, cord blood will be collected at the time of delivery (if possible), a small piece of cord tissue and placenta will be collected and sampled as well (if available), You will be asked to self-collect a vaginal swab which will be used to assess the types of bacteria in your vagina at each visit. At delivery and if there are any signs of preterm birth, additional swabs will be gathered during a routine exam by your doctor. Shortly after your baby is born, two swabs will be collected from your baby's earlobe (this does not hurt at all). One blood sample will be also collected from your child between 1 and 3 days of age, and a second blood sample will be collected along with routine bloodwork between 4 and 8 weeks of age.

At each of your study visits the following will be done:

- General health questions will be asked
- Some specific questions regarding factors known to be associated with preterm birth, such as hygiene, sex and domestic violence will be asked
- A mouth swab will be collected
- Two vaginal swabs will be self-collected
- You will have blood drawn from an arm vein for routine laboratory tests including HIV viral load, CD4 cell count, routine chemistry, routine hematology, and lactate. At the same time as your routine tests 20 ml (4 teaspoons) of blood will be drawn to test for mtDNA quantity, quality, mtRNA, DNA length, mitochondrial proteins, vitamins, hormone levels and inflammation biomarkers (elements in the blood that show inflammation is

present). We will also test for a series of viral infections that are very common in humans and can be in the body for a long time with no symptoms if the immune system is healthy. We will test for viruses such as those that cause chickenpox (varicella zoster virus or VZV), herpes (herpes simplex virus or HSV), mononucleosis (Epstein Barr virus or EBV) as well as cytomegalovirus (CMV), and the virus formerly known as Hepatitis G (GB virus C or GBVC). This testing may include antibody (a protein in the blood made in response to a foreign substance or a toxin - like an infection) testing and viral DNA (molecules in the blood that carry the virus' genetic information) and viral RNA (molecules that carry the virus' instructions from the DNA into proteins) testing. Because we are using non-diagnostic methods of testing (for research use only), we will not be giving these results to you or your doctor.

Regarding your baby:

- Two earlobe swabs will be collected shortly after birth
- The amount of blood drawn will be approximately 5 drops, or $\frac{1}{4}$ teaspoon for the one scheduled with newborn bloodwork at about 2 days of life, and approximately 10 drops or about $\frac{1}{2}$ teaspoon for the one at 4 to 8 weeks of age.

The study visits **do** require a small amount of additional time over a usual clinic appointment. About 20-25 minutes at the first study visit and then 15-20 minutes at each of the other visits will be needed for study related activities. All of the scheduled blood work is routine except for the mtDNA quantity, quality, mtRNA, DNA length, mitochondrial proteins, vitamins, hormone levels, inflammation biomarker and viral infection; tests; these tests require 20 ml or 4 teaspoons of blood which will be drawn at the same time as the routine blood tests. The results of all the blood tests, mouth swabs, vaginal swabs and earlobe swabs will be charted in the study paperwork. The blood, cord tissue and placenta samples will be

stored for up to 25 years except in cases where the Optional Tissue Banking Consent has been signed and then tissues may be stored for an indefinite period.

Baseline information will be extracted from the clinical record and, if available, will include information such as: the ethnicity of both parents, maternal health history, current and prior pregnancy histories, maternal drug/toxic exposures, delivery and neonatal events.

RISKS AND/OR DISCOMFORTS

Risks from Blood Drawing

Blood drawing may cause some discomfort, bleeding or bruising where the needle enters the body. A small blood clot may form where the needle enters the body or there may be swelling in the area. Rarely, fainting or a local infection at the puncture site may occur.

You may find the domestic violence questions upsetting. If this happens for you, or if you screen as positive for domestic violence with our questions, we will connect you right away with the nursing staff at the clinic. The nurses will help you and further connect you with other [redacted] team members such as a social worker, a counsellor, an outreach worker or a physician.

The vaginal self-swabbing might cause a small amount of discomfort; however, by self-swabbing you can control the speed and location of the sample collection.

The earlobe swab is collected from the outer ear and is not painful at all for the baby.

We will make every effort to protect your privacy and confidentiality during the study; however, it is possible that people may learn that you are participating in the study, and this may make you uncomfortable.

There are no other risks associated with participation in this study.

BENEFITS

We will not be able to use any of the results from this study to tell you whether or not you are at an increased risk of these side effects or for preterm birth and you will not receive any direct benefit from these results. However, knowledge gained from this study may, in the future, help other pregnant women who suffer from HIV/AIDS and their children.

NEW FINDINGS

You will be told of any new information learned during the course of the study that might cause you to change your mind about staying in the study. At the end of the study, you will be told when study results may be available and how to learn about them.

VOLUNTARY PARTICIPATION

Your participation in this research study is strictly voluntary. You may choose not to participate in this study or to withdraw from participation in the study at any time without providing any reasons for your decision. It will not influence the availability or quality of your present or future health care at this facility.

Please take time to read this information carefully and to discuss it with your family, friends, and doctor before you decide.

COSTS AND REIMBURSEMENT

You will be paid $20.00 for each study visit to help with the cost of transportation, parking or childcare. No receipts are required for this and you will be paid at the time of each visit.
Dr [redacted] or any of the other doctors involved in the study will not receive any money for your participation in this study.

You should know that one of the investigators, Dr. [redacted] is an inventor on a patent that has been filed by the University of British Columbia, on the mtDNA test used in this study. Therefore, [redacted] and UBC could one day receive a financial benefit from this research. You have the right to request more information about this financial benefit. You will not be eligible to receive any additional financial benefit from participating in this study even if the test should become commercialized.

IN CASE OF RESEARCH RELATED INJURIES

Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else, and you do not release the study doctors or participating institutions from their legal and professional responsibilities.

CONFIDENTIALITY

Your confidentiality will be respected. You will be assigned a unique study number as a participant in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity [i.e. your name or any other information that could identify you] as a participant in this study will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law.

Your tissue samples will be stored in a deep-freezer at the [redacted] at the [redacted]. The freezer is located in a locked room which is further located in the [redacted] which is locked after hours and on weekends. The custodian of these samples is Dr[redacted]. Samples are batched and tests are run in batches for quality assurance. All tissue samples are identified with your study ID only and will be stored for up to 25 years, except in cases where the Optional Tissue Banking Consent has been signed and then tissues may be stored for an indefinite period.

No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor.

ADDITIONAL INFORMATION

If you have any questions or need more information about this study at any time, please contact [redacted], or the study coordinator, [redacted]

If you have any concerns or complaints about your rights as a research participant and/or your

experiences while participating in this study, contact the Research Subject Information Line in the University of British Columbia Office of Research Services by e-mail at [redacted] or by phone at [redacted]

PARTICIPANT CONSENT

- ☐ I have read and understood the participant information and consent form.
- ☐ I have had sufficient time to consider the information provided and to ask for advice if necessary.
- ☐ I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- ☐ I understand that all of the information collected will be kept confidential and that the result will only be used for scientific objectives.
- ☐ I understand that my participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in any way the quality of care that I receive.
- ☐ I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- ☐ I understand that this study will provide no specific benefit to me.
- ☐ I have been told that I will receive a dated and signed copy of this form.

I have read this form and I consent to participate in this study.

_____

Printed name of and signature of **participant**

Date

_____

Printed name and signature of person obtaining consent

Date

This consent was done in the following language:

_____

The person signing below acted as an interpreter/translator for the participant,

during the consent process.

_____

Printed name and signature of person assisting in consent discussion **Date**

B.: CARMA-1 & CARMA-PREG consent form for tissue banking

Mechanism of Aging Following Exposure to HIV Antiretroviral Drugs

CIHR Team Grant in HIV Therapy and Aging

CARMA-1: MITOCHONDRIAL AND TELOMERE STUDIES IN PREGNANCY

AND

Placental Mitochondrial Toxicity of HIV Therapy during Pregnancy:

Clinical Tool Development and Determination of Outcome Variables for Clinical Trials

AND

MEASURING MITOCHONDRIAL AGING, APPLICATION TO HIV INFECTION AND THERAPY

AND

CELLULAR AGING AND HIV COMORBIDITIES IN WOMEN AND CHILDREN

carma-1-preg

~ Optional Consent for Tissue Banking ~

~ All Subjects ~

Site Principal Investigator: [redacted]

Local CIHR Team Co-Investigators

Dr [redacted]	Dr [redacted]	Dr [redacted]	Dr [redacted]

	[redacted]	[redacted]	

Emergency contact 24 hrs/7 days/week [redacted]

INTRODUCTION

Throughout this consent form, when we say "you" or "your", we mean you or your child.

In addition to the main part of the research study, you are being invited to participate in this optional study because the samples you provided as part of the main study could be used to continue our research in this area. As more information becomes available on the effects of HIV medications we would like to have the opportunity to do further new laboratory studies on your tissues/ samples. You can choose to participate in the main study without participating in this optional part of the study.

This consent form will explain why we wish to keep your tissues/samples and what will happen to your tissues/samples after they are collected. Once you understand the study, if you agree to take part in the study, you will be asked to sign this consent form. You will be given a copy of this form to keep for your records. This

project is funded by a grant from the Canadian Institute of Health Research (CIHR).

BACKGROUND

Despite amazing advances that can reduce the transmission of HIV from mother to child from about 25% to less than 1%, HIV transmission remains one of the most devastating concerns in the world today. An estimated 20 million women of reproductive age around the globe are living with HIV and drugs to prevent transmission to the babies are readily available to only a small percent, mainly in the developed countries. However, access to therapy is rapidly expanding throughout the world and there needs to be careful assessment of the possible toxic effects that are only recently being understood. Our research project that you've already consented to participate in will study changes in mitochondrial DNA (mtDNA, an energy producing part of body cells), damage drugs may do to telomeric DNA (the length of DNA at the end of chromosomes) and factors that contribute to preterm birth. We believe that changes in mtDNA or in the telomeric DNA may impact people's health and even lifespan and may in fact be one of the contributors to preterm birth.

PURPOSE

The reason we want to keep your sample stored is that this field of research is changing very rapidly and new discoveries are made often. If we keep your sample, we will be in a position to apply new tests that are not available today but would be in the future. Of course, any new test that we would apply to your sample would be geared toward the same research goal, the study of the effects and possible toxicity of HIV drugs on humans, and how this may affect their long-term health. For example, these may include:

- new technologies to analyze the quantity of mtDNA
- new technology to analyze the genetic coding in mtDNA
- new technology to analyze damage to mtDNA
- new technologies to analyze telomeric DNA damage
- new technologies to analyze markers of inflammation
- other tests that would reveal changes in the sample that are associated with aging or disease

WHO CAN PARTICIPATE?

This optional part of the study is open to women and their children enrolled in the main part of the study.

WHAT DOES THIS PART OF THE STUDY INVOLVE?

This part of the study does **not** involve any more of your time or the collection of any additional blood or information from you.

STUDY PROCEDURES / TISSUE BANKING DETAILS

- Your blood, small pieces of your placenta and cord tissue will have been collected as part of the main study (the amount of each is described in the main consent).
- The tissues (blood, placenta and cord tissue) will be stored at [redacted] at [redacted].
- The tissue samples at [redacted] have **none** of your personally identifying information on them; they are identified with your main study ID number only.
- Your name, as a part of the main study, is on a password-protected electronic master list kept on a password-protected computer network in a locked research office.
- Dr [redacted] is the custodian of the tissue samples for as long as they are stored.
- [redacted], the [redacted], is the custodian of the study master list.
- The tissues will be tested as outlined in the main consent.
- It is possible that tests yet to be developed or identified, but which are related to the tests being done in the main study could become available in the future. It is in the event of an opportunity such as this that the research team requests your consent to store and test your tissues for an indefinite amount of time.
- Should the research team want to conduct any future tests on your tissues that are NOT directly related to the main study tests (mtDNA quantity, quality, mtRNA, DNA length, mitochondrial proteins, vitamins, hormone levels and markers of inflammation), this would only be done after you are invited to participate and choose whether or not to give your consent.
- You will not be notified of the results of the tests in the main study; the exact relevance of these tests and how they might impact your medical care

is not known.

- If you choose to enter the study and then decide to withdraw yourself at a later time, all data collected about you during your enrolment in the study and up to the time of withdrawal will be retained for analysis. By law, this data cannot be destroyed.
- If you decide to withdraw yourself from this part of the study you can choose to either have your tissue samples destroyed OR you can choose to have all of the study identification removed (in this case the tissue could still be stored and tested but never linked back to you).
- Only investigators who are part of the main study or collaborating with the study investigators will have access to your banked tissue.
- If at any time you wish to have your tissues destroyed, you can contact [redacted] who will personally ensure your wish is respected.

RISKS AND/OR DISCOMFORTS

There are no other known risks associated with participation in this part of the study.

BENEFITS OF PARTICIPATING IN THIS STUDY

There is no direct benefit to you from taking part in this part of the study.

We hope that the information learned from this study can be used in the future to benefit other women with HIV/AIDS and who may wish to become pregnant.

ALTERNATIVES TO PARTICIPATION IN THIS PART OF THE STUDY

The alternative is to not participate at all, and this will have no effect on your participation in the main study.

NEW INFORMATION

If new information arises during the research study that might affect your willingness to remain in the study, the research team will inform you of this information.

IF YOU DECIDE TO WITHDRAW YOUR CONSENT TO PARTICIPATE

Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this part of the study. Please take time to read the following information carefully and to discuss it with your family, friends, and doctor before you decide.

You can choose to withdraw your participation in this part of the study at any time without affecting your participation in the main study. You do not have to provide any reason for your decision not to participate nor will you lose the benefit of any medical care to which you are entitled or are presently receiving.

If you choose to enter the study and then decide to withdraw yourself at a later time, all data collected about you during your enrolment in the study and up to the time of withdrawal will be retained for analysis. By law, this data cannot be destroyed.

If you decide to withdraw yourself from this part of the study you can choose to either have your tissue samples destroyed OR you can choose to have all of the study identification removed (in this case the tissue could still be stored and tested but never linked back to you).

IN CASE OF RESEARCH RELATED INJURIES

Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else.

COSTS AND REIMBURSEMENT

There will not be any reimbursement for this part of the study.

[redacted] or any of the other doctors involved in the study will not receive any money for your participation in this study.

You should know that one of the investigators, Dr. Cote, is an inventor on a patent that has been filed by the University of British Columbia, on the mtDNA test used in this study. Therefore, she and UBC could one day receive a financial benefit from this research. You have the right to request more information about this financial benefit. You will not be eligible to receive any additional financial benefit from participating in this study even if the test should become commercialized.

CONFIDENTIALITY

You will be assigned a unique study number as a subject in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity [i.e. your name or any other information that could identify you] as a subject in this study will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected and also give you the right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor.

ADDITIONAL INFORMATION

If you have any questions or need more information about this study at any time, please contact Dr [redacted], or the study coordinator, [redacted].

If you have any concerns about your rights as a research subject and/or your experiences while participating in this study, contact the Research Subject Information Line in the University of British Columbia Office of Research Services by email at [redacted] Toll Free: [redacted]

SUBJECT CONSENT

- ☐ I have read and understood the subject information and consent form.
- ☐ I have had sufficient time to consider the information provided and to ask for advice if necessary.
- ☐ I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- ☐ I understand that all of the information collected will be kept confidential and that the result will only be used for scientific objectives.
- ☐ I understand that my participation in this part of the study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in any way my participation in the main study or the quality of care that I receive.
- ☐ I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- ☐ I understand that that this study will provide no specific benefit to me.
- ☐ I have been told that I will receive a dated and signed copy of this form.

I have read this form and I consent to participate in this study.

_____

Printed name and signature of **subject**

Date

_____

Printed name and signature of **person obtaining consent**

Date

This consent was done in the following language:

_____

☐ The person signing below acted as an interpreter/translator for the subject, during the consent process.

_____

Printed name and signature of person assisting in consent discussion **Date**

C.: MtDNA & Pregnancy study consent form (2004-2009)

The Effect of Antiretroviral Therapy on Maternal Blood Cell Mitochondrial DNA Levels During Pregnancy in HIV-Infected Women

And

Mitochondrial DNA Damage in Infants Exposed to HIV Antiretroviral Drugs in utero

~ mtDNA in Pregnancy ~ Informed Consent

~

~ Control Subjects ~

Principal Investigator: Dr [redacted]

[redacted]

Oak Tree Clinic Co-Investigators ~ [redacted]

Dr [redacted] Co-Director

Dr [redacted] Pediatrician

Dr [redacted] Co-Director

[redacted] Pharmacist

Co-Investigators

Dr [redacted]
[redacted]
[redacted],

Dr [redacted]
[redacted]
[redacted],

Dr [redacted]
[redacted]
[redacted]

Emergency contact 24 hrs/7 days/week ~ at

INTRODUCTION

You are being invited to participate in the research study, named above, because you are pregnant, healthy, are scheduled to have routine pregnancy blood work drawn and you have not been exposed to anti-HIV (human immunodeficiency virus) medications during your pregnancy. This study will take place at the Children's & Women's Health Centre of BC and is funded by grants from the Canadian Association for HIV Research (CANFAR) and Canadian Institute of Health Research (CIHR).

This consent form will provide you with information on the purpose of the study, how it may help you, any risks to you, and what is expected of you. Once you understand the study, if you agree to take part in the study, you will be asked to sign this consent form. You will be given a copy of this form to keep for your records.

PURPOSE

It has been shown that some anti-HIV drugs, as an unwanted side effect, may have a toxic effect on the cells of the body. Some of the medications can have an effect on different body systems that leads to mitochondrial (energy-producing part of body cells) dysfunction. When the mitochondria are not working properly (mitochondrial toxicity) the body can start to build up high levels of lactate (a byproduct of cell function). Some doctors have expressed concerns that mitochondrial toxicity may be even more common in pregnant women than in other adults on these medications. Also, when mitochondria are affected, they make molecules (small particles called free radicals) that can cause damage to DNA.

The purpose of this study is to study the effect of taking anti-HIV drugs during pregnancy using two new laboratory tests. One is for mitochondrial DNA (mtDNA) and will test the level of function of the mitochondria and the other test will look at damage drugs may do to the length of DNA at the end of chromosomes.

In order to properly examine the possible impacts of these drugs, comparisons need to be made to women who did not take anti-HIV medications during pregnancy. Your participation in this study would be as part of the control group, which is used to compare against the study group.

STUDY ELIGIBILITY/SCREENING

In order to be eligible to participate, you must:

6. Be 19 years of age or greater.
7. Be up to 24 weeks into your pregnancy (gestational age of 0-24 weeks).
8. Not be HIV-infected.
9. Not have been exposed to anti-HIV drugs during your pregnancy.
10. Not have known or suspected mitochondrial disease.
11. Be willing to have blood drawn at the Women's Health Centre three times prior to delivery.

You are **not eligible** to participate in the control group if you:

3. Are less than 19 years old.
4. Have a confirmed diagnosis of HIV-infection.
5. Have been exposed to anti-HIV drugs.
6. Have a known or suspected mitochondrial disease.

STUDY ENTRY & Schedule of visits

If you decide to take part in this study, and you sign this consent form, you are consenting to the following:

- 6 ml (approximately 1 teaspoon) of blood will be collected at the same time, when possible, as your routine pregnancy tests. The samples will be collected once in the first 4.5 months of your pregnancy (ideally between 16 and 20 weeks of pregnancy), at 24-28 weeks when ususal glucose testing is done and at 32-36 weeks when pre-delivery hemoglobin is normally tested. Cord blood, a small sample of the placenta will be collected at the time of delivery (if possible), and one blood sample will be collected from your child between 1 and 3 days of age at the same time that routine newborn blood work is collected. The amount blood drawn from your baby will be approximately 5 drops, or $\frac{1}{4}$ teaspoon.
- Your and your infant's samples will be tested for lactate, mtDNA quantity, quality, mtRNA level, DNA length and mitochondrial proteins. Additionally your sample will be tested for platelets (if not already scheduled by your family doctor).
- The placenta sample will be destroyed after these tests have been performed
- Baseline information will be extracted from the clinical record and, if available, will include information such as: the ethnicity of both parents, mother's health history, current and prior pregnancy histories, maternal drug/toxic exposures, delivery and neonatal events.

Participation in the control group of this study will involve about 30-45 minutes of additional time, in which to read the consent, have any questions answered, and to have blood taken. The amount of blood requested from control participants is 6 ml (approximately 1 teaspoon) of blood at each visit. The results of all the blood tests will be charted in the study paperwork.

RISKS AND/OR DISCOMFORTS

Risks from Blood Drawing

Blood drawing may cause some discomfort, bleeding or bruising where the needle enters the body. A small blood clot may form where the needle enters the body or there may be swelling in the area. Rarely, fainting or a local infection at the puncture site may occur.

There are no other risks associated with participation in this study.

BENEFITS

Taking part as a control in this study offers you no direct benefit. However, knowledge gained from this study may, in the future, help other women who suffer from HIV/AIDS and become pregnant.

VOLUNTARY PARTICIPATION

Your participation in this research study is strictly voluntary. You may choose not to participate in this study or you may withdraw form participation at any time, without providing reasons for your decision and it will in no way influence the availability or quality of your present or future health care at this facility.

COSTS AND REIMBURSEMENT

You will be paid $20.00 for each study visit to help with the cost of transportation, parking or childcare. No receipts are required for this and you will be paid at the time of

each visit. Dr Money will not receive any money for your participation in this study. You should know that one of the investigators, Dr. Cote, is an inventor on a patent that has been filed by the University of British Columbia, on the mtDNA test used in this study. Therefore, she and UBC could one day receive a financial benefit from this research. You have the right to request more information about this financial benefit.

IN CASE OF RESEARCH RELATED INJURIES

Signing this consent form in no way limits your or your child's legal rights against the investigators, or anyone.

CONFIDENTIALITY

Your and your child's confidentiality will be respected. No information that discloses your or your child's identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you or your child may be inspected in the presence of the Investigator or his or her designate by representatives of, Health Canada, and the UBC Research Ethics Board for the purpose of monitoring the research. However, no records, which identify you or your child by name or initials, will be allowed to leave the Investigators' offices.

ADDITIONAL INFORMATION

If you have any questions or need more information about this study at any time, please contact Dr [redacted] at [redacted], or the study coordinator, [redacted] RN at [redacted]

If you have any questions or concerns regarding your treatment as a research subject, please contact the Research Subject Information Line in the UBC Office of Research Services at [redacted]

SUBJECT CONSENT

I understand that my participation in this study is entirely voluntary and that I may refuse to participate or withdraw from the study at any time without any consequence to my continuing medical care.

I have received a signed and dated copy of this consent form for my records.

I consent to participate in this study.

_____

Printed name and signature of **subject**

Date

_____

Printed name and signature of **witness**

Date

_____

Printed name and signature of **investigator**

Date

D. SOP Drug treatments for Cell culture experiments

SOP

Drug treatments for Cell culture experiments

This protocol will detail the steps for treating both adherent and suspension cells with 1 Cmax of antiretroviral (ARV) drugs each that are used in combination antiretroviral therapy (cART)

1. Prepare 10,000 Cmax master stocks (freezer stock) of individual ARVs. Detailed information for 10,000 Cmax stock preparation can be found in the excel sheet labelled "Drug info for treatments_MASTER".

   *Note: For Efavirenz (EFV) and Lopinavir (LPV), maximum stock that can be made is 3000 Cmax and 4000 Cmax, respectively (due to extremely low solubility of these drugs)

2. Prepare stocks of 2000 Cmax, 3000 Cmax and 4000 Cmax in 100% DMSO from master stocks of each drug.

For dual therapy (usually NRTI backbone):

From 2000 Cmax stocks of the two drugs to be used, take 2 µL of each drug and make up to a final volume of 4 mL in media (of the specific cell line).

i.e. 2 µL of 2000 Cmax Drug A + 2 µL of 2000 Cmax Drug B → total volume of 4 mL media

= 1 Cmax of each drug at 0.1% DMSO

The addition of the combined 4 µL of two drugs to 4 mL media should be done sequentially to gradually dilute out the DMSO. This should be done as follows:

Step 1: 4 µL of drugs (2 µL+ 2 µL) → 36 µL media = **40 µL** → 100 Cmax of each drug in 10% DMSO

Step 2: 40 µL → 360 µL media = **400 µL** → 10 Cmax of each drug in 1% DMSO

Step 3: 400 µL → 3.6 mL media = **4 mL** → **1 Cmax of each drug in 0.1% DMSO**

Deliver required volume of media containing drugs (eg. 3 mL for 6 well plates) to wells

For suspension cells (eg. CEM): Take 300 µL of this dilution and add it to 2.7 ml of media containing cells (already seeded in plates), i.e.

300 µL of 10 Cmax dilution, 1% DMSO → 2.7 mL media w/cells = 3 mL → 1 Cmax in 0.1% DMSO

For triple therapy (all except LPV/r):

From 3000 Cmax stocks of the three drugs to be used, take 2 µL of each drug and make up to a final volume of 6 mL in media (of the specific cell line).

i.e. 2 µL of 3000 Cmax Drug A + 2 µL of 3000 Cmax Drug B + 2 µL of 3000 Cmax Drug C → total volume of 6 mL media

= 1 Cmax of each drug at 0.1% DMSO

The addition of the combined 6 µL of three drugs to 6 mL media should be done sequentially to gradually dilute out the DMSO. This should be done as follows:

Step 1: 6 µL of drugs (2 µL+ 2 µL + 2 µL) → 54 µL media = **60 µL** → 100 Cmax of each drug in 10% DMSO

Step 2: 60 µL → 540 µL media = **600 µL** → 10 Cmax of each drug in 1% DMSO

Step 3: 600 µL → 5.4 mL media = **6 mL** → **1 Cmax of each drug in 0.1% DMSO**

Deliver required volume of media containing drugs (eg. 3 mL for 6 well plates) to wells

For suspension cells (eg. CEM): Take 300 µL of this dilution and add it to 2.7 ml of media containing cells (already seeded in plates), i.e.

300 µL of 10 Cmax dilution, 1% DMSO → 2.7 mL media w/cells = 3 mL → 1 Cmax in 0.1% DMSO

For triple therapy (with LPV/r):

From 4000 Cmax stocks of the four drugs (LPV and RTV are separate) to be used, take 2 µL of each drug and make up to a final volume of 8 mL in media (of the specific cell line).

i.e. 2 µL of 4000 Cmax Drug A + 2 µL of 4000 Cmax Drug B + 2 µL of 4000 Cmax LPV + 2 µL of 4000 Cmax RTV → total volume of 8 mL media

= 1 Cmax of each drug at 0.1% DMSO

The addition of the combined 8 µL of four drugs to 8 mL media should be done sequentially to gradually dilute out the DMSO. This should be done as follows:

Step 1: 8 µL of drugs (2 µL+ 2 µL + 2 µL + 2 µL) → 72 µL media = **80 µL** → 100 Cmax of each drug in 10% DMSO

Step 2: 80 µL → 720 µL media = **800 µL** → 10 Cmax of each drug in 1% DMSO

Step 3: 800 µL → 7.2 mL media = **8 mL** → **1 Cmax of each drug in 0.1% DMSO**

Deliver required volume of media containing drugs (eg. 3 mL for 6 well plates) to wells

For suspension cells (eg. CEM): Take 300 µL of this dilution and add it to 2.7 ml of media containing cells (already seeded in plates), i.e.

300 µL of 10 Cmax dilution, 1% DMSO → 2.7 mL media w/cells = 3 mL → 1 Cmax in 0.1% DMSO