Therapy-Related Hepatic Mitochondrial Dysfunction in Patients Co-Infected with Human Immunodeficiency Virus and Hepatitis C Virus and in HepG2 Cells

by

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Abstract

Background: Co-infection with HIV and hepatitis C virus (HCV) worsens liver disease and decreases highly active antiretroviral therapy (HAART) tolerability. HAART usually includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a protease inhibitor (PI) or a non-NRTI (NNRTI). NRTIs, particularly D-NRTIs, can induce mitochondrial DNA (mtDNA) depletion, deletions or mutations and lead to mitochondrial dysfunction. Multi-drug resistance protein-1 (MDR1) transports drugs across cellular membranes and may modulate toxicity. This project investigated HAART-related mitochondrial toxicity in liver tissue from HIV/HCV co-infected individuals and in human hepatic (HepG2) cells.

Hypotheses:

1) Patients ON-HAART will have altered pathology scores, mtDNA quantity/deletions and mt-mRNA/MDR1-mRNA levels compared to patients OFF-HAART, and these will be influenced by type of HAART.

2) Treatment with the d-NRTI didanosine (ddI) and the PI saquinavir (SAQ) will alter HepG2 cell viability, population doubling time (PDT) and mtDNA content.

Methods: Double-liver biopsies were collected from HIV/HCV co-infected individuals. One sample was used to score pathology, the other to extract DNA and RNA. mtDNA quantity, mt-mRNA and MDR1-mRNA levels were investigated by quantitative-PCR and mtDNA deletion by long-template PCR. Measurements were compared between individuals ON- versus OFF-HAART, on D-NRTI versus other NRTIs and on PI versus NNRTI.

HepG2 cells were exposed to ddI and SAQ. Cell viability, PDT and mtDNA content were investigated.
**Results:** Individuals ON-HAART (N=34) were similar in age, gender and HCV genotype to those OFF-HAART (N=18), and the groups did not differ significantly in pathology score, mtDNA quantity/deletions or mt-mRNA/MDR1-mRNA levels. The same was true for individuals on D-NRTI (N=6) versus other NRTIs (N=28) and on PI (N=17) versus NNRTI (N=8), except that individuals on PI were older (p=0.044) with higher mt-mRNA levels (p=0.015).

Treatment of HepG2 cells with ddI lowered mtDNA content while SAQ decreased PDT. Addition of a second drug (SAQ or ddI) exacerbated these effects. ddI transiently decreased viability.

**Conclusions:** The lack of differences between the ON- and OFF-HAART groups supports previous observations that HAART is not associated with increased hepatic mitochondrial toxicity although the cell culture findings suggest complementary toxicity upon co-exposure to ddI/SAQ.

This study may inform management of HIV/HCV co-infected individuals.
Preface

Ethics approval for the clinical study was issued by the following board: University of British Columbia – Providence Health Care Research Ethics Board (UBC-PHC REB).

The certificate number is H03-50055.
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<tr>
<td>min</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MSM</td>
<td>Men having sex with men/unprotected homosexual sexual activity</td>
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between men

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<td>mtDNA</td>
<td>Mitochondrial deoxyribonucleic acid</td>
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<td>mt-mRNA</td>
<td>Mitochondrial messenger ribonucleic acid</td>
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<td>mtRNA</td>
<td>Mitochondrial ribonucleic acid</td>
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<td>nDNA</td>
<td>Nuclear deoxyribonucleic acid</td>
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<tr>
<td>NEL</td>
<td>Nelfinavir</td>
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<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
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<td>NVP</td>
<td>Nevirapine</td>
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<td>Protease Inhibitor</td>
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<td>Joint United Nations Programme on HIV/AIDS</td>
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<td>World Health Organization</td>
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Lastly, I am grateful to my loving and supportive family, particularly my husband and my mother. I am also thankful for my son, Jonah, who was born partway through my degree.
Dedication

To my family
1.1 Human immunodeficiency virus (HIV) epidemiology

Human immunodeficiency virus (HIV) is an infectious virus that causes acquired immune deficiency syndrome (AIDS). Since the initial identification of AIDS in the United States in 1981 and the subsequent identification of HIV in 1983, the virus has infected over 60 million people and AIDS has caused more than 25 million deaths around the world \(^1\). There were approximately 34 million people worldwide living with HIV at the end of 2010, according to the most recent update from the Joint United Nations Programme on HIV/AIDS (UNAIDS) \(^2\). HIV is most prevalent in developing countries, particularly sub-Saharan Africa where according to UNAIDS, 67% of global HIV infections and 72% of global AIDS-related deaths occur \(^2\). There are two genotypes for HIV that have been characterized, 1 and 2, with genotype 1 being responsible for most of the global AIDS pandemic and genotype 2 being responsible for a small number of infections in West Africa \(^3\).

Fortunately, the global incidence of HIV has generally declined since the peak of the epidemic in 1997 \(^1\). \(^2\), in part due to improved global access to HIV antiretroviral (ARV) therapy and safer sexual behaviour. However, despite this overall global decrease in the incidence of new HIV infections and a decrease in HIV-related deaths, there are still almost two million deaths related to the virus each year and it remains one of the major global causes of premature death \(^2\).

In Canada, there were approximately 65,000 people infected with HIV at the end of 2008, according to the most recent update by the Public Health Agency of Canada (PHAC) \(^4\). This represents a 14% increase in incidence from PHAC’s last most recent estimate of 57,000 in 2005. Despite the global decrease in new HIV infections since
1997, the incidence of HIV in Canada has been steadily increasing since 1981. Aboriginal people are over-represented in the HIV-infected population in Canada, representing 8% of the infected population and only 3.8% of the Canadian population.

Twenty-one percent of HIV-infected Canadians reside in British Columbia (BC) despite the fact that the province accounts for 13% of Canada’s total population. The number of individuals infected with HIV in BC is therefore disproportionally high compared to the rest of the country. This makes studying HIV in the BC population particularly relevant.

1.2 HIV pathophysiology

HIV belongs to the retrovirus family of viruses and contains ribonucleic acid (RNA). The unique pathophysiology of HIV is discussed in detail below, including its replication cycle, routes of transmission and the natural progression of HIV to AIDS.

1.2.1 Replication cycle

Receptor-mediated fusion at the host cell membrane initiates replication of HIV (Figure 1). An envelope glycoprotein (gp120) that is present on the surface of the HIV virion binds to specific cluster designation 4 positive lymphocyte (CD4) receptors present on the surface of certain host cells. The majority of CD4 receptor-expressing cells are T helper lymphocytes, macrophages and dendritic cells, all of which have functions in the human immune system. Dendritic cells are the most probable route of entry into the uninfected individual, since they are widespread in mucosal membranes and are responsible for antigen presentation to lymphocytes. An HIV-bound dendritic cell then travels to a lymph node where the HIV virion infects a CD4 T lymphocyte. The high-affinity binding between gp120 and the CD4 receptor triggers a
conformational change in gp120 that displaces a portion of the glycoprotein so that it may act as a binding site for one of two specific co-receptors (CCR5 and CXCR4)\(^6\). Once bound, the viral envelope fuses with the host cell membrane and the viral core is inserted into the host cell cytoplasm\(^6\). The viral core consists of two copies of a single-stranded RNA molecule that encodes nine proteins, including reverse transcriptase (RT), integrase, protease and several other viral proteins\(^10\).

**Figure 1: HIV Replication Cycle**

The HIV virion fuses to the host cell membrane and the viral core is inserted into the cytoplasm. The viral RNA molecule is reverse-transcribed by viral RT into a double-stranded DNA molecule. The DNA molecule enters the host cell nucleus and the DNA is incorporated into the host’s nuclear genome. The viral genome is transcribed by the host cell’s machinery and new viral RNA molecules are produced, some of which function to make a polyprotein that is cleaved into individual proteins. These proteins, along with a new genomic RNA molecule, move to the surface of the host cell and a new HIV virion is formed. The new virion buds out and leaves the cell.

Following its entry into the cell, the RNA molecule is reverse-transcribed by the
viral RT into a double-stranded DNA molecule. Next, the DNA molecule enters the host cell nucleus via a nuclear pore and the DNA is incorporated into the host’s nuclear genome via the viral integrase enzyme. The newly-incorporated viral genome is then transcribed by the host cell’s machinery and new viral RNA molecules are produced, some of which function to make a polyprotein that is then cleaved into individual proteins via the viral protease. These proteins, along with a new genomic RNA molecule move to the surface of the host cell and a new immature HIV virion is formed. The new HIV virus buds out and eventually leaves the cell, forming a new HIV particle that may enter into a new host cell. Rather than being transcribed into new HIV RNA molecules, the double-stranded HIV proviral DNA in some cells may settle into a non-replicating latent phase. Reactivation of latent proviruses may occur at a future time once transcription is initiated by the viral transactivator protein, Tat.

1.2.2 Transmission

HIV is transmitted via transfer of bodily fluids, including blood, semen, vaginal fluid, and breast milk. This occurs when a mucosal membrane (vaginal, rectal or oral) or the punctured skin (referred to as the parenteral route) of an uninfected person comes into contact with a body fluid of an HIV-infected individual. The most common risk factors for contracting the disease are unprotected sexual activity, intravenous drug use (IDU) and perinatal exposure. In developing countries, most HIV infections occur through unprotected heterosexual sexual activity (HET). There are increasing numbers of exposures from HET in developed countries, compared to the past where HIV transmission was largely from unprotected male homosexual sexual activity or men having sex with men (MSM) and IDU.

In 2008 in Canada, 48% of HIV infections were transmitted from MSM, 17% from
IDU, 31% from HET, 3% from MSM and/or IDU, and 1% from other causes (such as occupational exposure, perinatal exposure and persons who contracted the virus but reported no identified or known risk) \(^4\) (Table 1). Transmission among Aboriginal people in Canada is unique, with 66% of HIV infections among this population as a result of IDU \(^4\). In BC in 2009, 47% of HIV infections were transmitted from MSM, 22% from IDU, 12% from HET, and 19% from other causes \(^5\) (Table 2).

**Table 1: Prevalence of HIV in Canada by Group**

<table>
<thead>
<tr>
<th></th>
<th>MSM</th>
<th>IDU</th>
<th>HET</th>
<th>MSM/IDU</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>N 31,330</td>
<td>11,180</td>
<td>19,960</td>
<td>2,030</td>
<td>500</td>
<td>65,000</td>
</tr>
<tr>
<td>%</td>
<td>48</td>
<td>17</td>
<td>31</td>
<td>3</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2005</td>
<td>N 27,700</td>
<td>10,100</td>
<td>16,910</td>
<td>1,820</td>
<td>470</td>
<td>57,000</td>
</tr>
<tr>
<td>%</td>
<td>48</td>
<td>18</td>
<td>30</td>
<td>3</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

Ref \(^7\)

**Table 2: Prevalence of HIV in BC by Group**

<table>
<thead>
<tr>
<th></th>
<th>MSM</th>
<th>IDU</th>
<th>HET</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>N 6,418</td>
<td>3,042</td>
<td>1,667</td>
<td>2,559</td>
<td>13,686</td>
</tr>
<tr>
<td>%</td>
<td>47</td>
<td>22</td>
<td>12</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>N 6,255</td>
<td>2,961</td>
<td>1,574</td>
<td>2,561</td>
<td>13,351</td>
</tr>
<tr>
<td>%</td>
<td>47</td>
<td>22</td>
<td>12</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

Ref \(^5\)

1.2.3 Natural disease progression

The typical natural course of HIV disease progression begins with what is referred to as the primary or acute infection phase. This phase normally lasts 2-8 weeks and is
characterized by rapid viral replication leading to a marked increase in HIV RNA in the circulation, up to several million copies of HIV RNA per mL of blood \(^{18, 19}\). This is accompanied by a transient yet significant decrease in circulating CD4 T lymphocytes (levels return to normal approximately 2-4 months post-infection), the main target of HIV virions \(^{20}\). However, the HIV RNA level decreases once CD8 (cytotoxic) T lymphocytes begin to attack HIV virions and this results in antibody production and seroconversion, and a positive HIV test \(^{21}\). Eventually, a steady state is reached, whereby the generation of new HIV virions is matched by their destruction by CD8 cells \(^{22}\). A rapid CD8 response to HIV is associated with slower disease progression and better overall prognosis \(^{20, 23}\). Two to 4 weeks post-infection, roughly half of all newly-infected individuals will develop influenza-like symptoms that last between a week and a month \(^{24, 25}\). Since these symptoms are similar to those of more common viruses, the most infectious phase often goes unnoticed and undiagnosed \(^{26}\).

The second phase of natural HIV progression is the latent or asymptomatic phase that typically lasts several years. During this chronic stage of the disease, there is a gradual decrease in CD4 T lymphocytes and a gradual increase in HIV RNA copy number \(^{7}\). During this phase, HIV replication typically occurs at a rapid rate of \(10^8\) virions per day in the individual’s lymphoid organs \(^{27}\), which are the site of the majority of lymphocytes. The decline in CD4 cells is due to increased cell death as a result of rapid HIV virion replication. As well, CD8 T lymphocytes reach levels that are above normal and remain elevated until the final stage of HIV infection \(^{21}\).

The third and final phase of natural HIV progression is the symptomatic phase or AIDS. This occurs when CD4 levels reach 200 cells per µl or less and the viral load increases in the peripheral blood \(^{7, 21}\). Homeostasis of immune cells is typically lost approximately 24 months prior to the onset of AIDS \(^{28}\). During this phase, there is
severe loss of immune function and increased susceptibility to opportunistic infections. Without treatment, median survival from time of infection is 10-11 years

1.3 HIV treatment: Highly active anti-retroviral therapy (HAART)

In 1987, zidovudine, the first HIV antiretroviral (ARV) drug, was introduced to the market. In the early 1990s, several more ARV drugs became available. At that time, HIV was treated with mono- and dual-ARV therapy, which often led to the development of HIV drug resistance and ultimate treatment failure. In 1996, a second class of HIV ARVs was introduced and since then, HIV is treated with a combination of drugs, referred to as highly active antiretroviral therapy (HAART). Today, there are over 25 ARVs used in HIV therapy. HAART has been successful in attaining undetectable viral loads (less than 50 HIV RNA copies per mL of plasma), improving CD4 counts, decreasing opportunistic infections and morbidity, and increasing patient survival. Decreased HIV plasma viral load (pVL) also results in the benefit of decreased infectiousness of the virus, thus lowering the risk of transmitting HIV to another individual. However, despite the great success of HAART, there is currently no cure for HIV, due to HIV integration into the host’s nuclear genome, and it is managed as a lifelong chronic disease. The WHO currently recommends that HAART be initiated in an HIV-infected individual once their CD4 T cell count decreases to 350 cells per µl or less, unless co-infection with tuberculosis or hepatitis B virus is present or if pregnant, in which case HAART should be initiated regardless of CD4 count. According to the WHO, HIV infected individuals who are co-infected with hepatitis C virus (HCV) should follow the same protocol as HIV mono-infected individuals. However, more recently, the Department of Health and Human Services published their latest guideline that says there is strong evidence for treating
HIV infected individuals with a CD4 count of 500 cells per µl or less with HAART, while moderate evidence shows that HAART can be considered for those with a CD4 count above 500 cells per µl, regardless co-infection with other pathologies. This same panel recommends that HAART be administered to all HIV/HCV co-infected individuals and that HAART may be used in conjunction with HCV antiviral therapy.

HAART typically consists of three drugs from a minimum of two drug classes. There are currently six main HAART classes, each targeting a step in the replication cycle of HIV (Table 3): entry or fusion inhibitors (EIs and FIs), nucleo(s/t)ide RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), integrase inhibitors (IIs), and protease inhibitors (PIs). EIs target the entry of the virion into the host cell. FIs target fusion of the HIV virion to the host cell membrane. NRTIs and NNRTIs target HIV RT and inhibit transcription of HIV DNA from HIV RNA. IIs target HIV integrase and inhibit HIV DNA integration into the host’s nuclear genome. Finally, PIs target HIV protease and inhibit HIV polyprotein cleavage.
Table 3: HAART Drug Targets and Potential Off-Target Effects

<table>
<thead>
<tr>
<th>HAART Class</th>
<th>Target</th>
<th>Potential Off-Target Effects</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Els</td>
<td>C-C chemokine receptor type 5 (host)</td>
<td>Hepatotoxicity, malignancy</td>
<td>39, 40</td>
</tr>
<tr>
<td>FIs</td>
<td>HIV envelope glycoprotein gp41</td>
<td>Injection site reactions including erythema, indurations, nodules, cysts, pain</td>
<td>41, 42</td>
</tr>
<tr>
<td>NRTIs</td>
<td>HIV reverse transcriptase</td>
<td>Hepatotoxicity, mitochondrial toxicity, neuropathy, myopathy, lipodystrophy, hyperlactatemia, lactic acidosis</td>
<td></td>
</tr>
<tr>
<td>NNRTIs</td>
<td>HIV reverse transcriptase</td>
<td>Hepatotoxicity, cutaneous reactions, neuropsychiatric symptoms, metabolic disturbances, gastrointestinal toxicity</td>
<td>43, 44</td>
</tr>
<tr>
<td>IIs</td>
<td>HIV integrase</td>
<td>Hepatotoxicity, malignancy, gastrointestinal toxicity, hypercholesterolaemia, hypertriglyceridaemia, hyperbilirubineamia</td>
<td>45, 46</td>
</tr>
<tr>
<td>PIs</td>
<td>HIV protease</td>
<td>Hepatotoxicity, hyperlipidaemia, hypercholesterolaemia, hypertriglyceridaemia, insulin resistance, impaired glucose metabolism, dyslipidaemia, gastrointestinal toxicity</td>
<td>47, 48</td>
</tr>
</tbody>
</table>

1.3.1 Nucleotide reverse transcriptase inhibitors (NRTIs)

HAART normally involves the use of a class of drugs called NRTIs. NRTIs are modified nucleosides that inhibit viral RT and cause viral chain termination. NRTIs require phosphorylation by kinases to become triphosphorylated and active. NRTIs lack the 3’-hydroxyl group on the deoxyribose, which is the site of attachment of the phosphate group of subsequent nucleosides during DNA elongation. Once an NRTI is incorporated into the viral DNA, the next incoming nucleotide cannot form the phosphodiester bond leading to premature viral chain termination. NRTIs were the
first class of ARVs used in HIV therapy and remain the backbone of most HAART regimens.

The first HIV ARV was the NRTI zidovudine (AZT) that was released onto the market in 1987. This was followed by didanosine (ddI), zalcitabine (ddC) and then stavudine (d4T) in the early 1990s. Since then, many more NRTIs have been introduced for use in HAART for HIV. These include lamivudine (3TC), abacavir (ABC), entecavir (ETV), emtricitabine (FTC) and tenofovir (TFV). In many parts of the world, particularly in more developed countries, certain older NRTIs (ddI and d4T) are less commonly prescribed compared to the newer NRTIs due to evidence of drug toxicity (see section 1.3.1.1).

1.3.1.1. D-drugs: Stavudine (d4T) and Didanosine (ddI)

Certain NRTIs are referred to as D-drugs. They are dideoxynucleosides and they have been shown to be more toxic to individuals than other NRTIs. The D-drugs are ddI, ddC and d4T. The toxic effects of NRTIs are correlated with the kinetics of their incorporation by the human mitochondrial polymerase gamma. ddC, followed by ddI and then d4T have been shown to be the most toxic NRTIs on the market and, because of this, ddC is no longer in clinical use and ddI is starting to be more often excluded from HAART regimens in developed countries. d4T is the least toxic of the D-drugs and it is still prescribed in many parts of the world.

1.3.2 Protease inhibitors (PIs)

PIs bind to HIV protease and block cleavage of viral polyproteins into individual proteins that are required for HIV virion maturation and cleavage from the host cell. PIs currently approved for HAART for HIV are saquinavir (SAQ), ritonavir (RIT),
indinavir (IND), nelfinavir (NEL), lopinavir (LOP), atazanavir (ATA) and darunavir (DAR). PIs are currently one of the most commonly-used classes of ARV in HAART, normally used in conjunction with two NRTIs.

PI-containing HAART regimens may be a concern for individuals who are co-infected with HIV and HCV due to the possibility of PI-related hepatotoxicity. PIs are associated with concentration-dependent elevations in hepatic aminotransferases, an indication of decreased hepatic function, as well as unconjugated hyperbilirubineemia. PIs have also been shown to inhibit the activity of the cytochrome P450 (CYP) 3A4 isoenzyme responsible for the metabolism of many therapeutic drugs. PI inhibition of CYP 3A4 activity may result in higher concentrations of the other therapeutic drugs in the body, leading to hepatotoxicity and decreased hepatic function.

1.3.3 Non-nucleotide reverse transcriptase inhibitors (NNRTIs)

NNRTIs inhibit viral RT via allosteric inhibition. NNRTIs bind to viral RT, close to the NRTI binding site, and deform the enzyme’s active site, preventing polymerization of DNA thus viral replication. NNRTIs currently approved for HAART are nevirapine (NVP), delavirdine (DMP), efavirenz (EFV), etravirine (ETR) and rilpivirine (RPV). NNRTIs are used similarly to PIs, often used in conjunction with two NRTIs.

NNRTI-containing HAART regimens may be a concern for individuals who are co-infected with HIV and HCV due to the possibility of NNRTI-related hepatotoxicity. NNRTIs such as NVP are associated with a hypersensitivity reaction and resulting hepatic injury and hepatitis that may be accompanied by jaundice, rash and fever. Although the mechanism for development hepatotoxicity is unknown, the risk for developing NNRTI-related hypersensitivity increases steadily over time during the first
year of therapy. Additionally, NNRTIs are metabolized by cytochrome P450s in the liver and may therefore be involved in drug interactions with other therapeutic drugs in the body that are also metabolized by cytochrome P450s, leading to possible hepatotoxicity and decreased hepatic function.

1.4 HIV/ hepatitis C virus (HCV) co-infection epidemiology

According to the WHO, there were an estimated 130-170 million people worldwide living with chronic hepatitis C virus (HCV) in 2000. The incidence of global HCV is not well known because chronic infection is often asymptomatic. The WHO estimates that 2-3% of the world’s population has been infected with HCV, with the highest prevalence (5-10%) found in Africa, Latin America, and Central and Southeast Asia. There are an estimated 350,000 HCV-related deaths worldwide each year.

In 2007, there were approximately 3.5 million people infected with HCV in North America and an estimated 242,500 of these cases were in Canada, corresponding to a prevalence of 0.7%. In Canada, Aboriginal persons and incarcerated individuals, with rates of 3% and 19% respectively, are disproportionately overrepresented among people infected with HCV. In BC in 2006, there were an estimated 40,000 people infected with HCV and 100 deaths per year due to the virus.

HIV and HCV share common routes of infection (mucosal and parenteral), so that HIV/HCV co-infection is common among individuals with HIV. Approximately one-third of HIV-infected individuals in developed countries around the world are co-infected with HCV. Twenty percent of HIV-infected Canadians are co-infected with HCV while as many as 50-90% of HIV-infected Canadians who practice IDU are co-infected. Overall, 53% of individuals in BC with HIV and HCV tests available are HIV/HCV co-infected so that, like HIV, HCV co-infection is disproportionately high in BC, making
the study of co-infection with the two viruses in this population particularly relevant.

1.5 HCV pathophysiology

HCV is thought to have originated in the early 1900s and was first identified in 1989. It is a small, enveloped, single-stranded, positive-sense RNA virus of the Flaviviridae family of viruses. There are multiple HCV genotypes due to a high error rate of the HCV RNA-dependant RNA polymerase. In fact, there are so many HCV variants that it is considered a quasispecies rather than a conventional virus species.

There are six major HCV genotypes in the world, numbered 1 through 6 and several subtypes within each genotype. The RNA sequences of the various genotypes differ by approximately 30-35% and the sequences of the subtypes differ by approximately 20-25%. Genotypes 1, 2 and 3 are found around the world, whereas genotype 4 is found in the Middle East and Africa, genotype 5 in South Africa and genotype 6 in Southeast Asia. Genotype 1 is the most common genotype in North America, Canada and BC, accounting for over 60% of HCV infections, followed by genotype number 3 and then genotype 2, which are also found in these locations.

1.5.1. Replication cycle

HCV replicates mainly in the hepatocytes of infected individuals but may also replicate in peripheral blood mononuclear cells. HCV RNA has also been detected in B and T lymphocytes, monocytes, macrophages, Kupffer cells and dendrocytes, although it is unclear whether the virus is actually replicating in these cells or is only able to enter them. Each infected cell may produce upwards of 50 virions per day, resulting in rapid replication of the virus once infected. Figure 2 shows the HCV
replication cycle. Entry into the host cell occurs via complex interactions between HCV virions and multiple specific cell-surface molecules. Once inside the host cell, the HCV virion employs the host cell machinery for replication. The single-stranded RNA molecule acts as an mRNA molecule and is directly translated to produce a polyprotein that requires cleavage by proteases to become ten individual proteins. HCV RNA replication occurs via viral RNA-dependant RNA polymerase, which produces a negative-sense strand RNA intermediate. This negative-sense strand serves as a template for the production of new positive-sense strand RNA viral genomes. Replication occurs on intracellular lipid membranes, where the viral core protein induces lipid droplet redistribution towards the nucleus, in a microtubule- and dyenin-dependant manner. Lastly, new virions are endocytosed from the host cell, likely using the LDL secretory pathway.
The HCV virion enters the host hepatocyte via a complex multi-step process. The virus is uncoated in the cell cytoplasm and a single-stranded positive sense RNA molecule is translated into a polyprotein. The cleaved proteins are used for HCV replication that occurs on intracellular lipid membranes. A membranous web is formed and a negative sense strand is made that serves as a template for the replication of new positive sense strands. The new virus is assembled and endocytosed, likely using the host LDL secretory pathway.

1.5.2. Transmission

As mentioned earlier, HCV transmission occurs via the same routes as that of HIV, mucosal and parenteral, when a mucosal membrane (including nasal) or the punctured skin of an uninfected person comes into contact with an HCV-infected body fluid of another individual. However, HCV in blood is ten times more infectious than HIV in blood\textsuperscript{99}, so that HCV infection from parenteral exposures is more prevalent than mucosal. Sixty percent of new HCV infections are a result of sharing unsterilized
needles with an HCV-infected individual \(^{100}\), 2-8% are from unprotected sexual activity \(^{101}\) and the remaining infections are from occupational, haemodialysis and perinatal exposures.

In Canada, as in the rest of the world, IDU is the greatest risk factor for HCV, representing 58% of prevalent HCV infections \(^{74}\). Blood transfusions account for 11% of HCV infections in Canada, haemophilia patients for 0.3%, and 31% of infections are from other risk factors, including sexual, occupational, and perinatal \(^{74}\). In BC and Vancouver, IDU is also the most important risk factor for contracting HCV \(^{102}\).

1.5.3. Disease progression

Approximately 12-16% of individuals who contract acute HCV have spontaneous resolution of the disease and may never even know that they had become infected \(^{103}\). In these individuals, the virus is naturally eradicated from the body within several months post-infection \(^{104}\). Acute HCV is asymptomatic for most individuals \(^{104}\). The remaining 85-90% of HCV-infected individuals go on to develop a chronic form of the infection \(^{101}, 103\). Both acute and chronic HCV infection are diagnosed by anti-HCV and HCV RNA in the blood, and chronic HCV is most often accompanied by elevated alanine transaminase (ALT) \(^{105}\).

Once chronic HCV is established, spontaneous resolution is uncommon; however, complete viral eradication is possible for some individuals. Approximately 45% of individuals with chronic HCV genotype 1 and 65% of individuals with chronic HCV genotype 2 or 3 who initiate HCV therapy will have successful eradication of the virus, demonstrated by sustained virologic response (SVR) \(^{106}\). The median rate of SVR for HCV-infected individuals across all HCV genotypes is 54-56% \(^{107}\). Whereas 15-33% of chronically HCV-infected individuals will experience mild fibrosis 40 or more years post-
infection, 20-33% of individuals who develop chronic HCV will develop cirrhosis or hepatocellular carcinoma within 20 or more years post-infection, although some may develop cirrhosis in as little as three years \textsuperscript{108}. Hepatocellular carcinoma is a highly lethal form of cancer that eventually results in symptomatic decompensated disease and median eight month survival \textsuperscript{107, 108}. The WHO estimates that 1-5% of mono-infected individuals who develop chronic HCV will die from end-stage liver disease \textsuperscript{70}.

Hepatosteatosis is the abnormal accumulation of large vacuoles of triglycerides within hepatocytes. It occurs in approximately 74% of HCV-infected individuals with HCV genotype 3 versus only 48% of HCV-infected individuals with other HCV genotypes \textsuperscript{109-111}. In patients with HCV genotype 3, hepatosteatosis is likely caused through a direct cytopathic effect \textsuperscript{112}, whereas hepatosteatosis in patients with other HCV genotypes is believed to be related to host factors, including race, obesity, hyperglycemia, hyperlipidemia and HCV viral load \textsuperscript{113, 114}. In HIV/HCV co-infected individuals, hepatosteatosis is more frequent and more severe than in those mono-infected with HCV \textsuperscript{114-116}.

Co-infection with HIV accelerates the natural course of HCV progression \textsuperscript{101}, while the effect of HCV on HIV progression is controversial. Some have found no effect \textsuperscript{117} or a decreased progression of HIV in HCV co-infected individuals \textsuperscript{118} and others have shown increased progression to AIDS and increased AIDS-related deaths in HIV/HCV co-infected individuals, demonstrating a more rapid progression of HIV disease \textsuperscript{119}. Furthermore, HIV and HCV co-infected individuals treated with certain HAART regimens have shown increased risk of developing drug-induced liver injury \textsuperscript{120, 121}. 
1.6 HCV treatment

As is the case with HIV, there is currently no vaccine for the prevention of HCV infection. One of the most difficult challenges in finding a vaccine for HCV is the high rate of mutation of the virus. However, unlike HIV infection, HCV can be cured with antiviral therapy. Until recently, standard therapy for HCV involved the use of two drugs: ribavirin and pegylated-interferon-α -2a or -2b. Recently, however, the American Association for the Study of Liver Diseases recommended that serine protease inhibitors be added to therapy for HCV genotype 1. Serine proteases are required for cleavage of structural viral proteins into their active forms. Therapy duration and dosage of ribavirin depends on HCV genotype and response to therapy.

1.6.1. Mechanisms

Although the exact mechanism of action of ribavirin is not fully understood, several mechanisms have been demonstrated. Ribavirin suppresses synthesis of viral nucleic acid by inhibiting host cellular inosine monophosphate dehydrogenase, leading to decreased pools of guanosine nucleoside in infected cells. This leads to inefficient translation of viral transcripts, causing synthesis of viral RNA with aberrant 5’ cap structures on purine nucleotides. Ribavirin also suppresses viral RNA-dependant RNA polymerase activity, upregulates genes involved in interferon signalling and shows immunomodulatory activity that enhances CD4 responses. Ribavirin is a prodrug that, once activated, resembles a purine RNA nucleotide (adenosine or guanine, depending on the rotation so that ribavirin pairs well with both uracil and cytosine). As such, ribavirin can become incorporated into an elongating HCV genome and cause lethal mutations, resulting in viral chain termination.

Interferon-α -2a and -2b are immune regulators that enhance the host’s immune
response to HCV \textsuperscript{130, 131}. Pegylation refers to the use of polyethelyne glycol that slows the clearance of interferon, resulting in a longer-acting therapeutic effect \textsuperscript{132, 133}.

1.6.2. Response to HCV therapy

The primary goal with HCV antiviral therapy is viral eradication and SVR, defined as undetectable HCV RNA six months post HCV therapy completion \textsuperscript{134}. Transient virologic response or virologic relapse, conversely, is defined as undetectable HCV-RNA upon therapy completion with reappearance of HCV-RNA post HCV therapy completion \textsuperscript{135}. Non-responders are individuals who fail to achieve undetectable HCV-RNA levels after 24 weeks of therapy \textsuperscript{136}.

Response to therapy depends on HCV genotype and therapy duration is adjusted accordingly \textsuperscript{111}. Out of the three genotypes (1, 2, 3) found in Canada, infection with genotype 1 is more refractory to HCV therapy, so that therapeutic agents are employed for twice as long as with genotypes 2 and 3 \textsuperscript{137}. The current recommended duration of HCV therapy for genotype 1 is 48 weeks, whereas infections with genotypes 2 and 3 are treated for 24 weeks \textsuperscript{126}. However, when treating HCV genotype 1, if HCV RNA has not declined significantly after 12 weeks or is still detected after 24 weeks, therapy cessation is considered since unresponsiveness to the antivirals is likely to continue and pursuing therapy may cause more harm than benefit \textsuperscript{136, 138}.

HCV therapy is recommended for HIV-infected individuals if the benefits of therapy outweigh the risks of potential therapy-related toxicity \textsuperscript{139, 140}. Some of the factors that may influence response to HCV antiviral therapy are HCV genotype, levels of CD4 T lymphocytes, levels of HCV viremia and HCV disease stage \textsuperscript{140-142}. If low levels of CD4 T lymphocytes are observed, then patients should first receive HAART to manage their HIV infection \textsuperscript{101}. HCV therapy is more successful in patients with low
levels of HCV RNA and with no or minimal fibrosis.\textsuperscript{142}

In the mono-HCV infected population, complete eradication from HCV antiviral therapy is observed in about 54-63\% of individuals.\textsuperscript{107, 143} However, when HIV co-infection is present, cure rates significantly decrease. Only 14-29\% of HIV/HCV co-infected individuals with HCV genotype 1 will have successful eradication of HCV\textsuperscript{140, 144}, versus 30-41\% of HCV mono-infected individuals.\textsuperscript{123, 145} Due to improved responsiveness of genotypes 2 and 3 to HCV antiviral therapy, approximately 24-62\% of HIV/HCV co-infected individuals will have successful eradication of HCV.\textsuperscript{144, 146}, versus 93 and 79\% of HCV mono-infected individuals with HCV genotypes 2 and 3, respectively.\textsuperscript{123} Although HIV/HCV co-infected individuals have a decreased likelihood of attaining SVR with HCV therapy, there are still many individuals for whom HCV therapy will be beneficial so that HCV therapy is recommended.\textsuperscript{140}

1.6.3. Side-effects of HCV therapy

The most common side-effect of treatment with ribavirin is haemolytic anaemia. Ribavirin accumulates inside erythrocytes and impairs glutathione levels.\textsuperscript{134} Oxidative damage ensues, resulting in hemolysis.\textsuperscript{134} Most individuals who are given interferon therapy experience flu-like symptoms soon after therapy initiation and some experience psychiatric side-effects, including depression and anxiety.\textsuperscript{147} Gastrointestinal effects, including nausea, diarrhea and weight loss are also often observed.\textsuperscript{147} Interferon has also been shown to reduce neutrophil and platelet counts, often necessitating in a dose reduction.\textsuperscript{148} although increased susceptibility to infection and increased severe bleeding have not been observed as a result of these blood findings.\textsuperscript{149, 150}
1.7 Liver biopsy

For the past 50 years, liver biopsy and histological scoring have been considered the “gold standard” for diagnosis of hepatic fibrosis and cirrhosis in HCV \(^{151}\). However, due to its invasiveness, measurement error and high cost, the use of alternative methods for diagnosis of hepatic fibrosis in some HCV-infected individuals has been proposed \(^{152-155}\).

A major limitation of liver biopsy is sampling error, since a biopsy only samples a very small portion of the organ and hepatic lesions may be heterogeneously-distributed \(^{154, 156, 157}\). Intra- and inter-observer variability of pathologic interpretations is another limitation inherent to liver biopsies \(^{154, 157-159}\).

Common adverse effects from a liver biopsy include transient and moderate pain (experienced by 10-30% of individuals \(^{160, 161}\)) and anxiety \(^{162}\). More serious potential complications include haemoperitoneum (presence of blood in the space between the abdominal wall and the internal abdominal organs), biliary peritonitis (inflammation of the membrane that lines the abdomen) and pneumothorax (abnormal collection of air or gas in the space that separates the lung from the wall of the chest) \(^{163, 164}\). Although extremely unlikely, liver biopsy may result in death to the patient in 0.009-0.5% of cases \(^{161, 165}\), a risk which is significantly reduced by the use of guided ultrasound \(^{166, 167}\).

1.7.1. Ishak-Knodell pathology score

One of the most commonly used histopathological scoring systems for liver biopsies is the Modified Histological Activity Index (HAI) Ishak-Knodell (IK) pathology score \(^{168-171}\) that will be referred to as the IK pathology score from here onward. As part of this scoring system, the patient’s liver tissue is examined for severity and location of liver inflammation, cell death and structural damage \(^{172, 173}\). Type of cell death
(apoptosis versus necrosis) is also considered \(^{172, 173}\). With the IK pathology scoring system, liver tissue is given a total score out of 24 (0 = a completely healthy piece of liver and 24 = a much damaged piece of liver). The pathologist examines the tissue for periportal/periseptal hepatitis, also referred to as piecemeal necrosis (scored 0 to 4), portal inflammation (scored 0 to 4), focal lytic necrosis, apoptosis and/or focal inflammation (scored 0 to 4), confluent necrosis (scored 0 to 6) and architectural changes, fibrosis and cirrhosis (scored 0 to 4) \(^{172, 173}\).

1.7.2. Non-invasive methods for determination of hepatic fibrosis

Due to the limitations and risks of liver biopsy, several non-invasive approaches to examine hepatic fibrosis are currently being pursued. Possible alternatives to liver biopsy include examination of serum markers, genetic and imaging techniques \(^{154, 157, 160, 161, 164}\). Although these methods are less invasive than a liver biopsy, they also have inherent limitations and are not always an accurate indicator of fibrosis \(^{154, 159, 164, 174, 175}\).

There is a long list of serum markers that can help stage fibrosis (Table 4) \(^{154}\). Serum markers are commonly divided into two categories: direct and indirect. Direct markers are fragments of the liver matrix that are produced by hepatic perisinusoidal cells during remodelling of the extra-cellular matrix \(^{154, 164, 176}\). By contrast, indirect markers are molecules that are found circulating in the blood when liver inflammation or liver function impairment is present as well as molecules that are synthesized, regulated or excreted by the liver \(^{154, 164, 176}\). Serum markers of fibrosis are commonly used in combination with each other and may also be used in conjunction with an imaging technique \(^{154, 164, 176}\). Some authors suggest that certain combinations of serum biomarkers are more accurate predictors of patient mortality and morbidity than a liver biopsy \(^{176}\).
Imaging techniques measure hepatic stiffness using either transient ultrasound elastography (TE), acoustic radiation forced impulse (ARFI) or magnetic resonance imaging (MRI)\textsuperscript{162,164}. Hepatic fibrosis is accompanied by changes in the microstructure of the liver that are reflected by an increase in liver stiffness and a resulting alteration in blood flow\textsuperscript{154,164}. 
### Table 4: Serum Markers of Fibrosis

<table>
<thead>
<tr>
<th>Abbreviations/ Indices</th>
<th>Individual components</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Direct</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PICP</td>
<td>procollagen type I carboxy terminal peptide</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PIIINP</td>
<td>procollagen type III amino-terminal peptide</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MMPs</td>
<td>metalloproteinases</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TIMPs</td>
<td>tissue inhibitors of matrix metalloproteinases</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Indirect</td>
<td>ASTM/ALT ratio</td>
<td>53</td>
<td>100</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGA</td>
<td>prothrombin index, GGT, apolipoprotein A1</td>
<td>91</td>
<td>81</td>
</tr>
<tr>
<td>APRI</td>
<td>AST/platelet count</td>
<td>89</td>
<td>75</td>
</tr>
<tr>
<td>FibroSpect II</td>
<td>hyaluronic acid, TIMP-1, α2-macroglobulin</td>
<td>84</td>
<td>67</td>
</tr>
<tr>
<td>FibroTest/FibroSure</td>
<td>γ2 macroglobulin, γ2 globulin, γ globulin, apolipoprotein A1, γ-glutamyl transpeptidase, total bilirubin</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>FibroIndex</td>
<td>platelet count, AST, GGT</td>
<td>78</td>
<td>74</td>
</tr>
<tr>
<td>FibroMeter</td>
<td>platelet count, γ2 macroglobulin, AST, age, prothrombin index, hyaluronic acid, blood urea nitrogen</td>
<td>81</td>
<td>84</td>
</tr>
<tr>
<td>Forns</td>
<td>age, platelet count, GGT, cholestered levels</td>
<td>94</td>
<td>51</td>
</tr>
<tr>
<td>HepaScore</td>
<td>age, gender, bilirubin, GGT, hyaluronic acid, γ2-macroglobulin</td>
<td>63</td>
<td>89</td>
</tr>
<tr>
<td>FIB-4</td>
<td>platelet count, ALT, AST, platelet count, age</td>
<td>70</td>
<td>74</td>
</tr>
<tr>
<td>SHASTA index</td>
<td>Hyaluronic acid, AST, albumin</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>Simple test</td>
<td>age, hyperglycemia, body mass index, platelet count, albumin, AST/ALT</td>
<td>78</td>
<td>58</td>
</tr>
<tr>
<td>OELF/ELF</td>
<td>age, hyaluronic acid, N-terminal propeptide of type III collagen, TIMP-1</td>
<td>41</td>
<td>90</td>
</tr>
</tbody>
</table>

ALT = alanine aminotransferase, GGT = gamma-glutamyl transpeptidase

Ref. 154
1.7.3. Current guidelines for liver biopsy

Whereas in the past a liver biopsy was widely used to guide decisions related to HCV therapy, many physicians and scientists now argue that a liver biopsy is no longer justified for most HCV-infected individuals. Due to the risks and limitations of liver biopsy, and the existence of non-invasive alternative methods to determine liver fibrosis, a liver biopsy is currently not required for most HCV-infected individuals seeking a diagnosis or therapy options. The current Canadian guidelines, based on expert opinion rather than on published evidence, recommend a liver biopsy prior to HCV antiviral therapy initiation for individuals with HCV genotype 1 but not for individuals with HCV genotypes 2 or 3. The decision to have a liver biopsy for individuals with HCV genotype 1 who wish to initiate HCV antiviral therapy continues to be debated in the United States.

According to the BC guidelines that were last updated in 2004, if an HCV-infected individual clearly meets HCV treatment criteria, a liver biopsy is only recommended if the biopsy may influence the decision to treat or if there is concern about other hepatic pathology. HCV treatment criteria in BC are elevated ALT for a minimum of six months or presence of certain associated diseases such as HIV, age less than 50 with HCV genotype 2 or 3, or advanced liver disease even if presenting with normal or minimally-elevated ALT. Although a liver biopsy is not mandatory for HCV therapy initiation, a biopsy may be helpful for some individuals, particularly if ALT is normal or only minimally elevated. The Canadian Liver Foundation published a report in June 2010 suggesting that liver biopsy is “not sufficiently dependable” to justify using it for HCV antiviral therapy decisions.
1.8 Hepatotoxicity in HIV/HCV co-infection

Liver disease is the greatest non-AIDS related cause of mortality in the HIV-infected population\textsuperscript{182, 183}. The mortality rate due to end-stage liver disease rose from 3\% in 1991 to 50\% in 1999 in one HIV-infected population\textsuperscript{184}. This may be explained in part by a longer life expectancy in the HIV-infected population due to the success of HAART and in part by co-morbidities like viral hepatitis. Ninety-three percent of HIV-infected individuals who die of end-stage liver disease are co-infected with HCV\textsuperscript{185}.

1.8.1. Liver disease

As mentioned above, HCV infection is a common cause of both acute and chronic liver disease; it can lead to hepatosteatosis, fibrosis and cirrhosis, end-stage liver disease and hepatocellular carcinoma\textsuperscript{107, 108}. HCV has become one of the major causes of liver failure and transplant in developed countries\textsuperscript{186}. HIV/HCV co-infection is associated with more rapid fibrosis progression to cirrhosis\textsuperscript{101, 146}. The mechanism is thought to be multi-factorial, involving direct HIV and HCV viral effects on hepatocytes\textsuperscript{187}, decreased HCV-specific T lymphocyte responses due to HIV infection\textsuperscript{188}, HCV-related immune activation, increased hepatocyte apoptosis and immunologic dysregulation\textsuperscript{101}. Due to increased fibrosis, higher rates of hepatosteatosis, cirrhosis, end-stage liver disease, hepatocellular carcinoma and liver failure are seen in the HIV/HCV co-infected population compared to HIV- or HCV-mono infected individuals\textsuperscript{101, 146, 189, 190}. Response to both HIV and HCV antiviral therapy is affected by co-infection and poor liver condition.
1.8.2. HAART tolerance

HIV-infected individuals who are co-infected with HCV experience three times more HAART-related hepatotoxicity compared to HIV mono-infected individuals. However, the mechanism underlying the association between HCV infection and HAART-related hepatotoxicity is not well understood. Despite these findings, HAART is recommended in the HIV/HCV co-infected population, since it slows the development and progression of fibrosis in these individuals. Indeed, HAART has been shown to increase life-expectancy in HIV/HCV co-infected individuals.

1.9 Mitochondria

Mitochondria are double membrane-bound organelles found in eukaryotic cells that generate most of the cell's energy. Mitochondria produce energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS), and are also involved in other cellular processes including cell cycle control, cell growth, apoptosis, intracellular signalling, heat production and metabolism of amino acids, lipids, cholesterol and nucleotides. Mitochondrial mass or abundance varies widely by tissue type, based on the energy consumption of the cell. Hepatocytes, for instance have a very high mitochondria content due to high energy demand.

Mitochondria are composed of four compartments that carry out specialized functions: the outer membrane that controls molecular diffusion in and out of mitochondria and is involved in signalling with the endoplasmic reticulum, the intermembrane space that is similar in composition to the cell cytosol, the inner membrane where the electron transport chain and ATP production occurs, and the matrix where enzymes and mitochondrial DNA (mtDNA) are located. Mitochondria
have their own DNA, separate from the nuclear DNA, that encodes genes involved in OXPHOS \textsuperscript{202}. The mtDNA genome counts 16,569 base pairs, is circular and double-stranded \textsuperscript{202}. It encodes 37 genes, including 22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides that are essential for OXPHOS \textsuperscript{203}. mtDNA differs from nuclear DNA in several ways. mtDNA exhibits polyploidy, lacks introns, is maternally-inherited, is replicated by polymerase gamma, undergoes polycystronic replication of all genes and is heteroplasmic (multiple different mutated mtDNA genomes may exist alongside wild-type genomes within the same organism, tissue, cell or organelle) \textsuperscript{204,205}.

The electron transport chain has two mobile electron carriers (ubiquinone and cytochrome c) as well as five enzymatic complexes (numbered I – V) \textsuperscript{206}. The complexes are proton pumps that function with the electron carriers to transport electrons through the inner membrane while simultaneously pumping protons out of the mitochondrial matrix and into the intermembrane space, thereby creating a proton gradient \textsuperscript{206,207}. The energy created by the protons returning to the matrix is used to synthesise ATP via conversion of adenosine diphosphate by ATP synthase (Figure 3).
Figure 3: Production of ATP by the Electron Transport Chain

The electron transport chain contains 5 complexes (complex I – V) and 2 mobile electron carriers (ubiquinone and cytochrome c). A proton gradient is generated by shuttling electrons between complexes and pumping protons (H+) from the mitochondrial matrix into the inter membrane space. The protons then flow down their gradient through complex V back into the matrix and ATP is made from ADP. Potentially harmful ROS are produced as a by-product of the electron transport chain.

ADP = adenosine diphosphate, ATP = adenosine triphosphate, ROS = reactive oxygen species

1.9.1. Overview

Certain antiviral compounds are associated with mitochondrial toxicity that can lead to mitochondrial dysfunction.

1.9.1.1. NRTI-related mitochondrial toxicity

NRTIs are used in HAART because they inhibit HIV RT by mimicking endogenous nucleotides and causing viral chain termination, as described in section 1.3.1. However,
NRTIs are also used by polymerase gamma, inhibiting, to a lesser extent, the replication of mtDNA. There is a broad range of reported clinical consequences to NRTI-related mtDNA toxicity that affect neuromuscular, gastrointestinal, haematological, nephrological, and metabolic organs. Clinical symptoms from NRTIs include peripheral neuropathy, myopathy, cardiomyopathy, gastrointestinal steatosis, pancreatitis, anaemia, neutropenia, peripheral lipoatrophy, bone marrow toxicity, hyperlactatemia or lactic acidosis, liver failure and death. The mechanism of NRTI-related mitochondrial toxicity is thought to involve polymerase gamma.

1.9.1.2. Mitochondrial polymerase gamma

Polymerase gamma, which is distinct from the nuclear DNA polymerases alpha and beta, replicates mtDNA. Compared to the nuclear polymerases, polymerase gamma is more error-prone, has poorer repair mechanisms and is more similar to HIV RT, the target of NRTIs. Therefore, NRTIs can become incorporated into the mtDNA genome and contribute to mtDNA damage, including depletion, mutations, and deletions. Accumulated mtDNA damage can eventually result in mitochondrial dysfunction, which is associated with accelerated aging and a number of age-associated diseases.

1.9.1.3. Oxidative stress

During OXPHOS, approximately 2-5% of the oxygen consumed can be converted to reactive oxygen species (ROS). ROS is formed when some electrons leak out of enzyme complexes I and III and generate a superoxide anion (O$_2^-$) that can then be converted into hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH·), all of which are
ROS species. Although antioxidants and free radical scavenging enzymes remove most ROS, these can oxidize and damage DNA, proteins and lipids. Because mtDNA is located close to the site of OXPHOS where most ROS are generated and is not protected by histones, it is more susceptible than nuclear DNA to damage from oxidative stress. Furthermore, mtDNA has poor repair and proof-reading capacity so that oxidative stress can accumulate mtDNA damage, leading to mitochondrial dysfunction, something that can further promote the production ROS and lead to a vicious cycle.

1.9.2. Mitochondrial (mt) DNA and mtRNA quantity

Exposure to NRTIs is associated with changes in mtDNA and mtRNA quantity. The ability of certain NRTIs to inhibit polymerase gamma can alter mtDNA quantity, although this is likely not the sole mechanism underlying these changes since an NRTI's ability to inhibit the enzyme is not always correlated with its effects on mtDNA quantity. Changes in mtDNA quantity are tissue-, drug- and dose-dependent and this has been demonstrated both clinically and in cell culture.

Although the effects of NRTIs on mitochondrial gene expression have not been extensively examined, one author reported an increase in mtRNA quantity in K562 lymphoblastoid cells exposed to ZDV and d4T, particularly in cells treated with the lowest doses of the drugs. Interestingly, this was accompanied by decreased mtDNA quantity such that alterations in mtDNA and mtRNA quantity are not necessarily correlated. Similarly, a clinical study showed increased mtDNA quantity and decreased mtRNA quantity in ARV therapy-exposed HIV-negative infants born to HIV-positive mothers compared to HIV-negative infants born to uninfected women.
1.9.3. mtDNA quality

Some researchers hypothesize that NRTIs may be associated with alterations in mtDNA quality, as evidenced by mutations in the mtDNA sequence \(^{232, 233}\). It is hypothesized that inhibition of polymerase gamma by certain NRTIs may be associated with decreased polymerase gamma fidelity and increased mutations in the mtDNA genome, something that may contribute to mitochondrial dysfunction \(^{59, 60, 208}\). Another theory is that NRTI may cause accelerated mtDNA turnover resulting in clonal expansion of existing mtDNA mutations, rather than NRTIs causing mutagenesis \(^{234}\). Our laboratory recently reported more mtDNA mutations in HAART-exposed HIV-infected pregnant women compared to HIV-uninfected controls, and a similar trend in their uninfected infants \(^{233}\). However, it remains unclear whether this observation is related to HIV infection, to HAART or both.

1.10 Multi-drug resistance protein 1 (MDR1)

Multi-drug resistance protein 1 (MDR1) is a human membrane-associated glycoprotein that transports various molecules across extra- and intra-cellular membranes \(^{235}\) and influences drug pharmacokinetics \(^{235}\). Some antiretroviral drugs are transported by MDR1 \(^{236}\).

1.10.1. MDR1 and HAART

Most HAART regimens involve the use of two NRTIs plus one NNRTI or one PI, and all three of these classes of drugs have been shown to interact with MDR1 \(^{236}\) thus potentially influencing drug levels \(^{236, 237}\). While NRTIs have been shown to inhibit MDR1 \(^{238}\), PIs and NNRTIs induce it \(^{239}\). It is, therefore, unclear how different combinations of HAART drugs will interact with MDR1 and what effects this may have.
on drug metabolism and interactions between HAART drugs. Additionally, the relationship of induction of MDR1 to mitochondrial toxicity is mostly unknown. To our knowledge, any examination of MDR1 activity in HIV/HCV co-infected individuals would produce novel findings.

1.11 Cell culture and HAART

Since it is difficult to control for variables in a clinical setting, the effects of ARV drugs are often studied in vitro. This section will present an overview of the current knowledge about HAART drug exposure in a human liver cell line model, with respect to mitochondrial toxicity and its effects on mtDNA integrity.

1.11.1. Human hepatocellular carcinoma (HepG2) cells

Human hepatocellular carcinoma (HepG2) cells are widely used as a model to investigate hepatic drug toxicity. HepG2 is an immortalized cell line that was derived from the liver tissue of a 15-year-old adolescent Caucasian American male who had a well-differentiated hepatocellular carcinoma.²⁴⁰

1.11.2. HepG2 and HAART

HepG2 cells exposed to certain NRTIs show alterations in mtDNA quantity and this may be explained, at least in part, by NRTI-induced mitochondrial dysfunction.⁵⁶, ²²⁷, ²⁴¹. Decreased mtDNA quantity in HepG2 cells exposed to NRTIs has been reported by several authors, in the following order: ddC > ddI > d4T > ZDV > 3TC = ABC = TDF⁵⁶, ²⁴¹-²⁴³. Therefore, HepG2 cells exposed to D-drugs have decreased mtDNA compared to HepG2 cells exposed to other NRTIs, demonstrating increased mitochondrial toxicity from D-drugs compared to other NRTIs. The effects of NRTIs on
HepG2 cell mtDNA quality with respect to mtDNA deletions is unknown as are the effects of NRTIs on HepG2 cell mtRNA quantity. To our knowledge, the effects of PIs and/or NNRTIs on HepG2 cell mtDNA quantity and quality have not been investigated, with the exception of one group who showed increased mitochondrial mass with no alteration in mtDNA quantity in HepG2 cells exposed to EFV.\textsuperscript{244}
CHAPTER TWO: CLINICAL STUDY

2.1 Overview

There are many HIV/HCV co-infected people living in BC and the effects of HAART on hepatic mitochondrial toxicity are not fully understood. Additionally, it is unclear whether certain HAART regimens may be more or less toxic to hepatic mitochondria and what role MDR1 activity may play.

2.1.1. Objectives

The goal of this study was to investigate the relationship between IK pathology score and mtDNA content, mtDNA deletions, mt-mRNA gene expression and MDR1 gene expression, in liver biopsies from HIV/HCV co-infected subjects, and to:

i) Compare those ON versus OFF-HAART

ii) Compare those on a PI- versus an NNRTI-containing HAART regimen

iii) Explore the relationship with being on a D-drug (ddI or d4T) versus other NRTIs

2.1.2. Hypotheses

We hypothesized that, pre-HCV therapy, patients ON versus OFF-HAART would have higher IK pathology scores show differences in mtDNA content, mt-mRNA gene expression, mtDNA deletions or MDR1 gene expression. We also hypothesized that these would be influenced by the type of HAART regimen.
2.2 Materials and Methods

2.2.1. Study population

This was a prospective observational cohort study that was funded by a 5-year grant from the Canadian Institutes of Health Research (identification code: HOP-75347, title: Mitochondrial toxicity in HIV/HCV co-infection antiviral therapy, principal investigators: Hélène Côté and Valentina Montessori). The original grant sought to compare hepatic mitochondrial toxicity from HAART before and after HCV antiviral therapy among the HIV/HCV co-infected population in BC. However, for this sub-study, we investigated HIV/HCV co-infected patients only prior to initiating HCV therapy.

All protocols and procedures were approved by the University of British Columbia Providence Health Care Research Ethics Board (certificate number: H03-50055). All study participants provided written informed consent upon entering the study. In BC, at the time of this study, a biopsy may be collected from an HCV-infected individual if they wish to be considered for HCV therapy and if either they do not clearly meet the criteria for treatment initiation (see section 1.6) or a secondary pathology requires investigation 180.

The majority of the study participants were enrolled at The British Columbia Centre for Excellence in HIV/AIDS (BCCfE) at St. Paul’s Hospital in Vancouver. Three participants were enrolled at a physician’s office (Dr. John Farley) in the city of Vancouver (BC). Individuals who were scheduled to undergo a liver biopsy as part of their evaluation for HCV therapy were invited to participate in this study and have a second or “double” liver biopsy collected. All participants were given the opportunity to opt out of the study at any point after consenting and were asked whether they wished to proceed with the second biopsy after the first biopsy was collected. The first biopsy was collected for their routine pathological examination and the second biopsy was
used for laboratory research investigation. Therefore, a “research” liver biopsy was only obtained if the individual was already undergoing a liver biopsy, at the request of their physician, for the purpose of histopathological examination. The study’s inclusion and exclusion criteria were as follows:

**Inclusion criteria:**

i) HIV-infected

ii) Anti-HCV antibody positive and HCV RNA-positive

iii) Either HAART-naïve, off HAART for a minimum of 6 months, or on stable HAART for at least 6 months prior to the double liver biopsy

**Exclusion criteria:**

i) Presence of any other chronic liver disease (such as hepatitis B virus, metabolic liver disorders, autoimmune liver diseases)

ii) Presence of an opportunistic infection within one month prior to biopsy collection

iii) Pregnancy or planning pregnancy during the study period (between pre- and post- HCV therapy biopsy collection)

2.2.2. Liver biopsy

Ultrasound-guided liver biopsies were collected at St. Paul’s Hospital by a physician. Biopsies were scheduled at approximately 10 o’clock in the morning and study participants were asked to be fasting beginning the night before the procedure. A local anaesthetic was applied after which a spring-loaded needle biopsy gun (10 mm core, 18 gauge) was used to collect the liver biopsies. The needle was inserted twice into the patient’s liver. The first biopsy was immersed in formalin for histological examination by a pathologist. The second liver biopsy was given to a member of the laboratory research team (either a staff member or a graduate student) who proceeded
immediately to a laboratory within the BCCfE to begin processing of the liver tissue sample.

The needle biopsy was cut into four relatively equal-sized aliquots (approximately 1mm² x 5mm each) within a biosafety cabinet using a sharp, sterile blade. The first liver aliquot was placed in a microcentrifuge tube with approximately 1.2 mL of RNAlater® (QIAGEN, Mississauga, ON, Canada) and kept at 4ºC overnight. RNAlater® is a reagent that stabilizes the RNA structure in tissues and cells in order to minimize RNA degradation. The following morning, the RNAlater was pipetted out and the tube was transferred to a -80ºC freezer and stored for future use in mitochondrial mRNA (mt-mRNA) quantification. Two of the biopsy pieces were placed in two microcentrifuge tubes and immediately frozen at -80ºC for use in mtDNA quantification and future mtDNA mutation studies. The fourth piece of liver biopsy was placed in an electron microscopy fixative solution (1.5 mL glutaraldehyde + 3.5 mL water + 5.0 mL 0.2M sodium cacodylate) prepared fresh earlier that morning. The tissue in this solution was stored at 4ºC for a laboratory technician or graduate student to carry out the processing for electron microscopy.

2.2.2.1. Pathology scores

The first liver biopsy collected from study participants was scored by a pathologist. For 48 subjects, an IK pathology score was given. An alternate type of pathology score, called the Batts-Ludwig and Metavir pathology score, was given to one study participant. The remaining three study participants from whom a liver biopsy was obtained were not given a pathology score. For two of those, the biopsy collected was unsuitable for scoring while another was not given to a pathologist for scoring. Since the majority (93%) of study biopsies received an IK pathology score, this scoring
system was used in this study.

2.2.3. DNA and RNA extractions

Total DNA and RNA were extracted from the liver biopsy specimens that had been immersed in the RNAlater solution, using the Allprep DNA/RNA Mini Kit (QIAGEN, Mississauga, ON, Canada), according to the manufacturer’s protocol with the following modification: DNA was eluted with AE buffer (QIAGEN) rather than with the EB buffer (QIAGEN) provided in the kit. Tissue samples were homogenized using a rotor-stator Polytron PT2100 homogenizer (Kinematic AG, Switzerland). Between samples, the homogenizer was cleaned by immersing the blades in distilled water and turning on the homogenizer for 20 seconds (s), followed by 70% ethanol for 20 s, and then again in distilled water for another 20 s. Extracted DNA and RNA samples were stored at -80°C. Additionally, at the time of RNA and DNA extraction, protein was precipitated from the liver tissues via acetone precipitation. The protein precipitation was performed as outlined in QIAGEN’s supplementary protocol and the optional step to wash the precipitates with ethanol was also performed. Protein samples were resuspended in 5% sodium dodecyl sulphate.

2.2.4. mtDNA quantification

mtDNA was quantified using an assay that compares the copy number of a mitochondrial gene (cyochrome c oxidase subunit I (COX1)) relative to the copy number of a single-copy nuclear gene (accessory subunit of the polymerase gamma (ASPG) \(^ {245, 246}\). The mtDNA/nDNA ratio of COX1/ASPG is a measure of mtDNA content, or mitochondrial genome quantity per nuclear genome. The assay was performed on the liver total DNA by real-time polymerase chain reaction (PCR) in a
LightCycler® 480 (Roche Applied Science, Laval, QC) with a LightCycler®480 Probes Master kit (Roche Applied Science). The sequences of the primers and probes used in this assay are shown in Table 5. Each PCR reaction contained 2 μl of extracted DNA sample, 5 mM magnesium chloride (MgCl$_2$), 1 μM of forward and reverse PCR primer, 0.2 μM fluorescein probe and 0.4 μM LC Red 640 probe, for a total volume of 10 μl. The PCR conditions used were 95°C for 10 minutes (min), followed by 45 amplification cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 5 s. Fluorescence measurements were acquired after each annealing step, in order to monitor the change in DNA quantity throughout the PCR amplification process. The amplified PCR products were quantified using a standard curve that was generated using six serial dilutions (1:10) of a plasmid (Topo; Invitrogen, Burlington, ON) containing both the ASPG and COX1 fragments.
Table 5: Sequences of Primers and Probes Used in the mtDNA Quantification Assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX1</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-TTCCGCCGACCGTTGACTATT-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-AAGATTATTACAAATGCATGGGC-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’-GCCAGCCAGGCAACCTTCTAGG-FI-3’</td>
</tr>
<tr>
<td>Red$_{640}$ Probe</td>
<td>5’-LCre$_{640}$-AACGACCACATCTACAACGTTATCGTCAC-P-3’</td>
</tr>
<tr>
<td>ASPG</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-GAGCTGTTGACGGAAGGAG-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-CAGAAGAGAAATCCCGGCTAAG-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’-GAGGCCTGTTAGAGATCTGTCAGAGA-FI-3’</td>
</tr>
<tr>
<td>Red$_{640}$ Probe</td>
<td>5’-LCre$_{640}$-GGCATTTCCTAAGTGGAAGCAAGCA-P-3’</td>
</tr>
</tbody>
</table>

P = Phosphate, Fl = Fluorescein, LCRed$_{640}$ = Lightcycler Fluorophore Red$_{640}$

Two negative controls (one containing no DNA and the other containing no enzyme) and two internal controls were included in every run. For each DNA sample, both ASPG and COX1 were always run on the same PCR 96-well plate, with each gene in duplicate. The duplicates were only accepted if they were less than 20% different from each other and if they fell within the standard curve’s range. The mean of the duplicate copy number values for each gene was recorded as the DNA copy number for that sample. MtDNA content was expressed as the ratio of mtDNA/nDNA.

2.2.5. mtRNA quantification

cDNA was prepared from the extracted RNA samples using the Quantitect® RT kit (QIAGEN). One microgram of template RNA was used in each cDNA reaction and the
assay was performed as per the recommended protocol. The quantification procedure used for the cDNA was similar to that used for the extracted DNA samples (see section 2.2.4). The expression of the mtDNA-encoded mitochondrial gene COX1 was measured, as was that of the nuclear DNA-encoded gene cytochrome c oxidase subunit 8 (COX8). PCR products were again quantified using a standard curve generated by 10-fold serial dilutions of Topo plasmid DNA (Invitrogen) containing COX1 and COX8, respectively. The mRNA PCR products of each gene were normalized to the mRNA level of the house-keeping gene beta-actin (β-actin) (COX1 or COX8 mRNA/β-actin mRNA). The primers and probes used for the cDNA quantification are summarized in Table 6.
Table 6: Sequences of Primers and Probes Used in the mt-mRNA Quantification Assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX1</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’- TTCGCCGACCGTTGACTATT-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’- AAGATTATTACAAATGCATGGGC-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’- GCCAGCCAGGCAACCTTTCTAGG-FI-3’</td>
</tr>
<tr>
<td>Red$_{640}$ Probe</td>
<td>5’- LCRed$_{640}$-AACGACCACATCTACAACGTTCGTCAC-P-3’</td>
</tr>
<tr>
<td>COX8</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’- CGCCAAGATCCATTGTTG-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’- CCAAGGAGGTCACCATGGAG-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’- CCCTGGATCATGTCAATTCCAG-FI-3’</td>
</tr>
<tr>
<td>Red$_{640}$ Probe</td>
<td>5’- LCRed$_{640}$-CACCTCTTTCTGCAATCATGACCTTTGA-P-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’- TCCTATGTGGGCGACGAGG-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’- GGTGTTGAGGTCTCAAACATG-3’</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5’- CCCRTGCTGCTGACCRAGGCC-FI-3’</td>
</tr>
<tr>
<td>Red$_{640}$ Probe</td>
<td>5’- LCRed$_{640}$-CCTGAACCCCAAGGCACCGY-P-3’</td>
</tr>
</tbody>
</table>

P = Phosphate, Fl = Fluorescein, LCRed$_{640}$ = Lightcycler Fluorophore Red$_{640}$

2.2.6. mtDNA deletions

For each DNA sample, the entire mitochondrial genome was amplified in two overlapping mtDNA fragments (Figure 4) by long-template PCR reactions, using the Expand Long Template PCR Kit (Roche). Extracted DNA was diluted to 100 copies of nDNA (ASPG), as determined by qPCR, in 5 μL with the same AE buffer that was used to elute the extracted DNA (QIAGEN). Each PCR reaction contained 5 μL of DNA
template, 0.35 mM dNTP, 0.3 M of each primer and 2.5 units of Expand Long Template polymerase (Roche), for a total volume of 50 μL. All primers were obtained from IDTDNA (Coralville, IA, USA) and the PCR amplification was performed using a MyCycler thermocycler (Bio-Rad, Hercules, CA, USA). The PCR reaction conditions used were 93°C for 2 min, followed by 10 cycles of 93°C for 10 s, 58°C for 30 s and 68°C for 6 min, followed by 25 amplification cycles of 95°C for 10 s, 58°C for 30 s and 68°C for 6 min and 20 s. The primers and probes used for the cDNA quantification are summarized in Table 7. A negative control with water in place of the template DNA was run each time the long PCR was performed. PCR products were separated on a 0.75% agarose gel by electrophoresis and stained with ethidium bromide. Gel images were captured under an ultraviolet light using a digital camera (Canon Inc., Tokyo, Japan). Deletions were quantified using gel densitometry software (UN-SCAN-IT, Silk Scientific, Orem, UT, USA). Two boxes were drawn for each sample: one around the deleted products (underneath the full-length band), and another that encompassed both the deleted bands and the full-length product, and the pixels in each box recorded. MtDNA deletion score was expressed as the ratio between deleted and total (full-length + deleted) PCR product.
Figure 4: Long-template PCR Amplification of mtDNA Fragments

The mitochondrial genome is amplified in 2 overlapping fragments (A and B) and the products are separated by electrophoresis. The bands at the top of the gel are full-length products while the bands farther down the gel are smaller length products that represent deleted products. The amount of mtDNA deletion present was estimated as the ratio of the density of deleted bands compared to the total density of the deleted bands and the full-length product. Fragments A and B were added for the final amount.

Table 7: Sequences of Primers and Probes Used in the mtDNA Deletion Assay

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-GCCACACGTTCCCTAATAAGA-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-CGGTAGTATTTAGGGGGCATTTAC-3’</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5’-CTCCTTGACGTTGACAATCGAGT-3’</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5’-GGGGATGCTTGCATGTGTAATCTTAC-3’</td>
</tr>
</tbody>
</table>
2.2.7. MDR1 quantification

The same cDNA that was prepared for the mtRNA assay (section 2.2.5) was used for the MDR1 quantification assay. Two different genes were used for this assay. MDR1 was the gene of interest and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene. Quantification of MDR1 was performed by comparing the copy number of MDR1 to the copy number of GAPDH (MDR1 mRNA/GAPDH mRNA). The primers and probes used for the cDNA quantification are summarized in Table 8. PCR was performed using a LightCycler® 480 (Roche Applied Science) and a LightCycler® 480 SYBR Green I Master Kit (Roche Applied Science) was used for the PCR reactions. PCR conditions used were 95°C for 10 min, followed by 45 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 5 s. Samples were run in duplicates on the same plate and the duplicates were only accepted if they were less than 20% different from each other.
Table 8: Sequences of Primers and Probes Used in the MDR1 mRNA Quantification Assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5'-GCTGGGAAGATCGCTACTGA-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-GGTACCTGCAAAACTCTGAGCA-3'</td>
</tr>
<tr>
<td>Probes</td>
<td>LightCycler 480 SYBR Green I Master (ROCHE, cat#04707516001)</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>5'-TTGGTATCGTGGAAGGACTCA-3'</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>5'-TGTCATCATATTTGGCAGGTTT-3'</td>
</tr>
<tr>
<td>Fluorescein Probe</td>
<td>5'-TGTCAGGACCTGACCTGCCGTCTAGA-P-3'</td>
</tr>
<tr>
<td>Red_{640} Probe</td>
<td>5'-LCRed_{640}-GGTGACCTGACCTGCCGTCTAGA-P-3'</td>
</tr>
</tbody>
</table>

P = Phosphate, Fl = Fluorescein, LCRed_{640} = Lightcycler Fluorophore Red_{640}

2.2.8. Statistics and data analysis

Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and XLSTAT (Addinsoft SARL). The Mann-Whitney test was applied to compare groups (ON vs. OFF HAART, PI vs. NNRTI, and D-drug vs. other NRTIs) for the following measurements: age, gender, HCV genotype, IK pathology score, mtDNA content, mt-mRNA gene expression, mtDNA deletions, and MDR1 gene expression.

2.3 Results

2.3.1. Study population

A total of 65 participants were enrolled in this study between July 2003 and May 2010: 53 males and 12 females (Figure 5). There were 13 lost to follow-up for the following reasons: five individuals could not be contacted for biopsy collection after
consenting to the study, two individuals opted out of the study and three individuals became deceased after consenting to the study and prior to biopsy collection. For two subjects, biopsies were collected for histopathological examination but no second biopsy was collected for laboratory research investigative purposes. One individual became ill and started using illicit drugs so that the subject was no longer a candidate for HCV therapy initiation and, therefore, a biopsy for pathological examination was no longer warranted. A total of 52 double liver biopsies were collected: N=43 male and N=9 female. All study subjects were infected with HIV genotype 1.

Figure 5: Clinical Study Participants
Sixty-five individuals provided written informed consent to join this study: 53 male and 12 female. Thirteen were lost to follow-up and a total of 52 double liver biopsies were collected: 43 male and 9 female, 34 ON HAART and 18 OFF HAART. Within the ON HAART group, 17 were on a PI and 8 were on an NNRTI while 6 were on a D-drug and 28 on other NRTIs.

At the time of biopsy retrieval, all of these individuals were being evaluated for potential initiation of HCV antiviral drug therapy and were undergoing a liver biopsy for histopathological examination at the request of their physicians. At the time of liver
biopsy collection, N=51 participants were HCV therapy-naïve. The characteristics of the study subjects are summarized in Table 9. According to the BCCDC and Health Canada, our study population reflects both the gender and HCV genotype proportions that currently exist within the HIV/HCV co-infected populations of both BC and Canada. All study participants reported being drug- and alcohol-free at the time of enrolment into this study.

Table 9: Characteristics of the Study Population: ON versus OFF HAART

<table>
<thead>
<tr>
<th></th>
<th>ON HAART</th>
<th>OFF HAART</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>34</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>82</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>HCV Genotype (1/2/3%)</td>
<td>76 / 0 / 24</td>
<td>71 / 5 / 24</td>
<td></td>
</tr>
<tr>
<td>CD4 Count (cells/µL)</td>
<td>395 [280-595]</td>
<td>380 [295-432]</td>
<td>0.60</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45 [44-53]</td>
<td>47 [40-49]</td>
<td>0.95</td>
</tr>
<tr>
<td>IK Pathology Score</td>
<td>7.5 [4.8-9.0]</td>
<td>7.0 [4.0-8.3]</td>
<td>0.84</td>
</tr>
<tr>
<td>mtDNA Content</td>
<td>368 [315-545]</td>
<td>399 [312-623]</td>
<td>0.81</td>
</tr>
<tr>
<td>Deletions</td>
<td>1.4 [1.0-1.9]</td>
<td>1.7 [1.5-4.0]</td>
<td>0.14</td>
</tr>
<tr>
<td>MDR1 Gene Expression</td>
<td>9.6 [5.9-15.2]</td>
<td>7.9 [6.1-10.3]</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Results expressed as median [IQR] unless otherwise specified. IQR = inter-quartile range, N = sample size

2.3.2. ON vs. OFF HAART

Thirty-four study participants were on stable HAART for a minimum of six months (and will be referred to as the ON HAART group), while 18 study participants were off HAART for a minimum of six months (and will be referred to as the OFF HAART group).
group). Of the 18 participants in the OFF HAART group, five were HAART-naïve. The number and proportion of study participants on each HAART drug is shown in Table 10. In our cohort, the two most commonly prescribed NRTIs were 3TC and TFV. The most commonly prescribed PI was RIT, however, this was taken in a low dose as a PI booster, so that the two most commonly prescribed PIs taken at regular doses were LOP and ATA. The two most commonly prescribed NNRTIs were DMP and NVP, and only one FI, ENF, and one II, RAL, were prescribed. The ON and OFF HAART groups had a similar proportion of males to females, with 82% male in the ON HAART group and 83% male in the OFF HAART group. Groups also had a similar proportion of HCV genotypes (1, 2 and 3), with a majority HCV genotype 1 (Table 9). No significant differences in participant age were noted (p=0.95) between the two groups, with a median age of 45 years in the ON HAART group and a median age of 47 years in the OFF HAART group. There were no significant differences in CD4 count between the ON and OFF HAART groups.
Table 10: Proportion of Study Participants on Each HAART Drug

<table>
<thead>
<tr>
<th>Drug</th>
<th>AZT</th>
<th>ddI</th>
<th>D4T</th>
<th>3TC</th>
<th>ABC</th>
<th>FTC</th>
<th>TFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>19</td>
<td>10</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>%</td>
<td>8.8</td>
<td>8.8</td>
<td>11.8</td>
<td>55.9</td>
<td>29.4</td>
<td>29.4</td>
<td>55.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>SAQ</th>
<th>RIT</th>
<th>IND</th>
<th>LOP</th>
<th>ATA</th>
<th>DAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2</td>
<td>20</td>
<td>2</td>
<td>9</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>%</td>
<td>5.9</td>
<td>58.8</td>
<td>5.9</td>
<td>26.5</td>
<td>29.4</td>
<td>5.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>NVP</th>
<th>DMP</th>
<th>ETR</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>%</td>
<td>17.6</td>
<td>23.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>ENF</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1</td>
</tr>
<tr>
<td>%</td>
<td>2.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>RAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
</tr>
<tr>
<td>%</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Results are shown as the number of participants on each drug and the percentage of total participants within the ON HAART group on each drug.

ENF = enfuvirtide, RAL = raltegravir

2.3.2.1. Pathology score

The ON and OFF HAART groups did not differ significantly in median IK pathology score (p=0.84). The ON HAART group had a median IK pathology score of 7.5 and the OFF HAART group had a median IK pathology score of 7.0.

2.3.2.2. mtDNA content and mt-mRNA gene expression

The ON and OFF HAART groups did not differ significantly in mtDNA content (p=0.81). The median mtDNA content ratios for the ON and OFF HAART groups were 368 and 399, respectively.
Similarly, the two groups had similar mt-mRNA gene expression ratios \((p=0.92)\). The ON HAART group had a median mt-mRNA gene expression ratio of 28 and the OFF HAART group had a median mt-mRNA gene expression ratio of 27.

2.3.2.3. mtDNA deletions

The ON and OFF HAART groups did not differ significantly in percent mtDNA deletions \((p=0.14)\). The ON and OFF HAART groups had percent mtDNA deletions of 1.4 and 1.7, respectively.

2.3.2.4. MDR1 expression

The ON and OFF HAART groups did not differ significantly in MDR1 gene expression \((p=0.35)\). The ON HAART group had a median MDR1 gene expression value of 9.6 and the OFF HAART group had a median MDR1 gene expression value of 7.9.

2.3.3. PI vs. NNRTI

Since no differences were seen between the ON and OFF HAART groups, we further investigated patients in the ON HAART group and compared patients who were on a PI-containing HAART regimen (on one or multiple PIs in addition to NRTIs) to those who were on an NNRTI-containing HAART regimen (on one or multiple NNRTIs in addition to NRTIs) (Table 11). Seventeen study participants were taking PIs while only eight study participants were taking NNRTIs. Seven study participants in the ON HAART group were on both a PI and an NNRTI and were thus excluded from this set of investigations. Two study participants in the ON HAART group were on neither a PI nor an NNRTI and were thus also excluded from this comparison. Therefore, a total of 25
study participants were compared here, however, data was not available for every participant for each parameter, so the number of participants who were actually compared is indicated for each measurement (Table 11). As mentioned previously, two IK pathology scores are missing because the medical pathologist was unable to score the biopsy collected. One mtDNA ratio is missing because the DNA extracted was too dilute and fell outside of the standard curve in the Real-Time PCR assay. Similarly, 8 mt-mRNA gene expression ratios and 12 MDRI values are missing due to insufficient cDNA such that the measures fell outside of the standard curve in the two Real-Time PCR assays.

Table 11: Characteristics of the Study Population: PI versus NNRTI

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PI</th>
<th>NNRTI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>17</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>88</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>HCV Genotype (1/2/3%)</td>
<td>71 / 0 / 29</td>
<td>75 / 0 / 25</td>
<td></td>
</tr>
<tr>
<td>Age (years)** Median [IQR], N</td>
<td>45 [42-52], 17</td>
<td>40 [39-41], 8</td>
<td>0.044</td>
</tr>
<tr>
<td>IK Pathology Score</td>
<td>8.0 [4.0-9.0], 17</td>
<td>6.0 [4.3-8.5], 6</td>
<td>0.74</td>
</tr>
<tr>
<td>mtDNA Content</td>
<td>391 [305-538], 17</td>
<td>320 [242-394], 7</td>
<td>0.19</td>
</tr>
<tr>
<td>mt-mRNA Gene Expression</td>
<td>34 [26-39], 13</td>
<td>17 [15-20], 4</td>
<td>0.015</td>
</tr>
<tr>
<td>Deletions</td>
<td>1.5 [1.0-1.9], 17</td>
<td>1.0 [0.9-1.2], 8</td>
<td>0.12</td>
</tr>
<tr>
<td>MDR1 Gene Expression</td>
<td>11.3 [8.6-15.2], 9</td>
<td>9.9 [7.5-12.9], 4</td>
<td>0.81</td>
</tr>
</tbody>
</table>

*IQR = inter-quartile range, N = sample size

The PI group was significantly older than the NNRTI group (p=0.044), with a
median age of 45 and 40 years in the PI and NNRTI groups, respectively. Participants on PIs versus NNRTIs had similar IK pathology scores (p=0.74). mtDNA ratios for the participants in the PI group did not differ significantly from the NNRTI group (p=0.19). The PI group had significantly higher (p=0.015) mt-mRNA gene expression compared to the NNRTI group, with median values of 34 and 17, respectively. The PI group and the NNRTI group did not differ significantly in percent mtDNA deletions (p=0.12) or in MDR1 gene expression (p=0.81).

2.3.4. D-drug vs. Other NRTIs

Since no differences were seen between the ON and OFF HAART groups, we investigated patients in the ON HAART group and compared patients who were on D-drugs versus other NRTIs (11). Among the study participants on D-drugs, three were on d4T, two were on ddI and one was on both. A total of 28 study participants were taking NRTI-containing HAART regimens that excluded d4T and ddI. All participants in both groups (D-drugs and other NRTIs) were also on either one or more PI, NNRTI, FI, or II. Therefore, 34 study participants were compared who were ON HAART and receiving at least one NRTI, however, data was not available for every participant for each parameter, as in section 2.3.3. The number of participants who were compared is indicated for each parameter (Table 12). Reasons for the missing values are the same as described in section 2.3.3.
Table 12: Characteristics of the Study Population: D-drug versus Other NRTIs

<table>
<thead>
<tr>
<th></th>
<th>D-drug</th>
<th>Other NRTIs</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>6</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>67</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>HCV Genotype (1/2/3%)</td>
<td>67 / 0 / 33</td>
<td>79 / 0 / 21</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>Median [IQR], N</td>
<td>Median [IQR], N</td>
<td>0.26</td>
</tr>
<tr>
<td>IK Pathology Score</td>
<td>47 [45-53], 6</td>
<td>44 [40-50], 28</td>
<td>0.79</td>
</tr>
<tr>
<td>mtDNA Content</td>
<td>357 [302-502], 6</td>
<td>368 [321-600], 26</td>
<td>0.68</td>
</tr>
<tr>
<td>mt-mRNA Gene Expression</td>
<td>35 [21-39], 5</td>
<td>27 [19-35], 19</td>
<td>0.57</td>
</tr>
<tr>
<td>Deletions</td>
<td>1.7 [1.3-2.4], 6</td>
<td>1.4 [0.96-1.9], 28</td>
<td>0.20</td>
</tr>
<tr>
<td>MDR1 Gene Expression</td>
<td>11.7, 2</td>
<td>9.6 [5.7-14.3], 14</td>
<td>0.81</td>
</tr>
</tbody>
</table>

IQR = inter-quartile range, N = sample size

No significant differences in participant age were noted between the two groups (p=0.26) (Table 12). Participants on D-drugs versus other NRTIs showed no differences in any of the parameters compared.
CHAPTER THREE: CELL CULTURE STUDIES

3.1 Overview

HepG2 cells have previously been used by several groups investigating the effects of HAART drug toxicity since they offer a model for what may be occurring in the livers of HIV-infected individuals. The effects of different HAART regimen exposures on HepG2 cell mitochondrial function is not fully understood, particularly exposing cells to two or more different classes of HAART drugs concurrently. As well, because OXPHOS is more essential when glucose stores are low, we investigated whether cells grown in low versus high glucose medium show differences in mitochondrial toxicity.

The HAART drugs chosen for our cell culture studies are d4T, ddl and SAQ. d4T and ddl were chosen because they are both D-drugs that have been shown to be more toxic to hepatic mitochondria in vitro compared to other NRTIs 56, 60, 227. The PI SAQ was chosen because it belongs to a drug class other than the NRTI class of drugs and many of the clinical study participants were on a PI, as opposed to an NNRTI, FI or II. Among PIs, SAQ is one of the older and more toxic drugs 68, 249 and so we thought we would see more of an effect versus using a newer and less toxic PI. To our knowledge, SAQ has never before been examined for hepatic mitochondrial toxicity in HepG2 cells, so that all findings related to SAQ in this cell line are novel.

3.1.1. Objectives

Three different experiments were performed, each with a different set of objectives.
**d4T experiment:**

i) To investigate how different concentrations of d4T (Cmax, 10 X Cmax, and 0.1 X Cmax) alter HepG2 cell viability, population doubling time (PDT; see section 3.2.6), mtDNA content and mt-mRNA gene expression.

ii) To compare the effects of d4T on HepG2 cell viability, PDT, mtDNA content, and mt-mRNA gene expression in high versus low glucose medium (4,500 mg/L vs. 1,000 mg/L).

**ddI experiment:**

i) To determine how reproducible mtDNA content and mt-mRNA gene expression findings are in HepG2 cells exposed to ddI (10 X Cmax).

ii) To determine mtDNA content and mt-mRNA gene expression in HepG2 cells treated with ddI and grown in high versus low glucose medium.

**ddI/SAQ experiment:**

i) To investigate the effects of adding one HIV drug (ddI or SAQ) and then adding a second class of drug (NRTI or PI) on HepG2 cell viability, PDT, and mtDNA content.

3.1.2. Hypotheses

Of the three different experiments that were performed, each sought to test a unique set of hypotheses.

**d4T experiment:**

i) d4T will decrease HepG2 cell viability, PDT and mtDNA content in a dose-and time-dependant manner.
ii) d4T will alter mt-mRNA gene expression, in a dose- and time-dependent manner.

iii) The above changes will be amplified in low glucose medium compared to high glucose medium.

**ddI experiment:**

i) ddI will decrease mtDNA content and alter mt-mRNA expression

ii) Variability between replicates of six will be less than 20%

**ddI/SAQ experiment:**

i) ddI will decrease HepG2 cell viability, PDT and mtDNA content.

ii) SAQ will alter HepG2 cell viability, PDT and mtDNA content.

iii) Addition of a second class of drug (NRTI or PI) may alter findings in parts i and ii.

3.2 Materials and Methods

3.2.1. Cell line

HepG2 cells were used in this study (product number: HB-8065, ATCC, Manassas, VA, USA). As specified by ATCC’s guidelines, cells were grown in an incubator at 37°C in 95% oxygen and 5% carbon dioxide.

3.2.2. Media

The normal base medium for HepG2 cells is Gibco®-formulated Dulbecco’s Modified Eagle Medium (product number: 30-2002, Life Technologies: Gibco, Burlington, ON). This media was used to harvest cells prior to experimentation. During
experiments, a high or low glucose medium was used (see section 3.2.2.1) as the base medium. Complete growth medium was made by adding foetal bovine serum (FBS) to a final concentration of 10% to the base medium. HepG2 cells are an adherent cell line, so cells would attach to the bottom of the flask during normal growth. This requires that cells be removed using trypsin from the bottom surface of the flask prior to cultivation (see section 3.2.4).

3.2.2.1. High vs. low glucose media

Since we are investigating mitochondrial function and the mitochondria perform OXPHOS, particularly in the absence of glucose (see section 1.2.1), we sought to promote oxidative phosphorylation by lowering the amount of glucose available to the cells in culture. This was accomplished by using a low glucose medium (product number: 11885, Gibco) with a glucose concentration of 1,000 mg/L. For a robust comparison to the low glucose medium, a high glucose medium was used (product number: 11965, Gibco) with a glucose concentration of 4,500 mg/L. To our knowledge, comparing high versus low glucose media with HepG2 cells treated with HAART has never before been published, so that all related outcomes are novel to the field.

3.2.2.2. Seeding determination

The number of cells that was seeded into the flasks for the d4T (3.2.3.1) experiment was determined by seeding several different known amounts of HepG2 cells in a 25cm² t-flask ((T25) Falcon BD: BD Biosciences, Franklin Lakes, NJ, USA) and observing them grow over several days. Since no data has been published on the growth rate of HepG2 cells in low glucose medium, we needed to determine how quickly cells would grow during the experiment. Once the desired confluency (60-80%)
was reached in an optimal time frame (3-4 days), we decided to seed with that number of cells in each flask for the experiment. We used six T25 flasks and seeded six different amounts of cells in the low glucose medium, as follows: $5.0 \times 10^5$, $1.0 \times 10^6$, $1.5 \times 10^6$, $2.0 \times 10^6$, $2.5 \times 10^6$, and $3.75 \times 10^6$ cells. Cell confluency was recorded visually on day 0 after 1 h and at the same time for the following 4 days (Figure 6). Four days post-seeding, the flask that had been seeded with $2.0 \times 10^6$ cells was approximately 70% confluent (estimated as described in section 3.2.4). Therefore, $2.0 \times 10^6$ cells were seeded in each flask for the d4T experiment, in both the low glucose and high glucose media, so that cells could be harvested approximately twice a week.

![Figure 6: Longitudinal HepG2 Cell Culture Confluency as a Function of Seeding Density](image)

Six different known amounts of HepG2 cells were seeded in low glucose medium. The flask seeded with $2.0 \times 10^6$ cells reached 70% confluency 4 days post-seeding, so this amount was chosen for the subsequent d4T experiments.
3.2.3. Subculturing technique

Confluency was estimated by observing flasks under a light microscope and estimating how much of the bottom surface of the flask was covered in a monolayer of cells. For the d4T and ddI/SAQ experiments, subculturing was performed when a cell culture flask reached between 60% and 80% estimated confluency. For the ddI experiment, cells were subcultured on specific days, as opposed to when a specific range of confluency was attained. All tubes and flasks used in the cell culture experiments were purchased from Falcon BD (BD Biosciences, Franklin Lakes, NJ, USA). The following subculturing procedure was followed:

1. The cell culture medium was removed using the vacuum attached to the biosafety cabinet and discarded.

2. To rinse away trypsin inhibitors contained in the serum, the layer of cells attached to the bottom of the flask were briefly rinsed with 4 ml of 0.25% (w/v) Trypsin-0.53mM EDTA solution (TrypLe, Gibco). After 20 s, the TrypLE was removed.

3. A volume of 0.5 ml of TrypLE was then added and the flask was placed inside the 37°C incubator for 10 minutes. This step was performed in order to help the cells detach from the surface of the flask. The flask was not shaken during this step to avoid damage from agitation.

4. A volume of 6 ml of complete growth medium was then added and cells were aspirated by gently pipetting up-and-down 20 times using a 10 ml automated pipettor tip.

5. Appropriate aliquots of the cell suspension were added to a new culture vessel. A total volume of 5 ml was used for 25 cm² culture t-flasks, and 2 ml in each well of the 6 well plates.

6. For freezing, cells were resuspended in complete growth medium with 5% (v/v)
DMSO and stored in the -80°C freezer until DNA and RNA extraction.

3.2.4. Treatments

HepG2 cells were exposed to multiple treatments, depending on the experiment being performed. Drug concentrations were based on the Cmax of the various drugs. The Cmax is the maximum drug plasma concentration measured following a routine therapeutic oral dose administration of the drug. Antiretroviral drugs were obtained in powder form from the National Institutes of Health AIDS Program (Maryland USA). The drugs were dissolved in either DMSO or PBS, depending on their solubility. d4T and SAQ were dissolved in dimethyl sulfoxide (DMSO) and ddI was dissolved in phosphate buffered saline (PBS). Fresh media containing drug was prepared and added to the cells every 48 to 72 hours, in order to ensure that the treatments did not lose their effectiveness. Each treatment was performed in duplicate in two different flasks. Control cells were cultured in medium with 0.02% PBS or 0.003% DMSO for the d4T experiment and 0.001% for the ddI/SAQ experiment, mimicking the concentration from added drugs.

3.2.4.1. Stavudine (d4T) experiment

The Cmax of d4T is 3.6 M\(^{250}\), so we rounded to 3 M so as to compare with what other authors have done\(^{60}\), and this concentration was used in addition to 10 X Cmax (30 M) and 0.1 X Cmax (0.3 M). Medium alone (both high and low glucose medium) was also used as a control in this experiment. In total, ten different treatment conditions were examined in this experiment, as shown in Table 13. Cells were treated with the various treatments for a total of 23 days and were collected when they reached 60-80% confluency.
Table 13: Treatment Conditions Used in the d4T Experiment

<table>
<thead>
<tr>
<th>Low glucose medium</th>
<th>High glucose medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium only</td>
<td>Medium only</td>
</tr>
<tr>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>0.3 M d4T</td>
<td>0.3 M d4T</td>
</tr>
<tr>
<td>3 M d4T</td>
<td>3 M d4T</td>
</tr>
<tr>
<td>30 M d4T</td>
<td>30 M d4T</td>
</tr>
</tbody>
</table>

3.2.4.2. ddl experiment

For this experiment, HepG2 cells were grown in either high glucose or low glucose medium and were treated with PBS or ddl, for a total of four different treatment conditions (Table 14). A concentration of 10 X Cmax of ddl was used. As the Cmax of ddl is $11.8 \mu M^{251}$, a drug concentration of $118 \mu M$ was used. Cells were exposed to the various treatments for a total of ten days and cells were collected on days three and ten. All treatment conditions were performed in replicates of six.

Table 14: Treatment Conditions Used in the ddl Experiment

<table>
<thead>
<tr>
<th>Low glucose medium</th>
<th>High glucose medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>118 M ddl</td>
<td>118 M ddl</td>
</tr>
</tbody>
</table>

3.2.4.3. ddl/SAQ experiment

For this experiment, HepG2 cells were treated with one HAART drug and then a second drug from a different class was added. The two drugs used in this experiment
were ddI, which is an NRTI, and SAQ, which is a PI. The Cmax of SAQ is 0.69 μM. Originally, 10 X Cmax was used for both drugs because we wanted to ensure that there would be a notable effect from the drugs on the cells. However, SAQ concentration was lowered to 1 X Cmax after excessive cell death was observed at the 10 X concentration. ddI was dissolved in PBS and SAQ was dissolved in DMSO, so both PBS and DMSO were used as control treatments in this experiment. Low glucose medium was used for all of the treatments in this experiment. In total, eight different treatment conditions were examined, as shown in Table 15. Cells were treated with the various treatments for a total of 21 days and all treatments were performed in duplicate. Cells were collected when they reached 60-80% confluency.

**Table 15: Treatment Conditions Used in the ddI/SAQ Experiment**

<table>
<thead>
<tr>
<th>Control Treatments</th>
<th>Drug Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (21 days)</td>
<td>ddI (21 days)</td>
</tr>
<tr>
<td>DMSO (21 days)</td>
<td>SAQ (21 days)</td>
</tr>
<tr>
<td>PBS (8 days), then</td>
<td>ddI (8 days), then</td>
</tr>
<tr>
<td>PBS + DMSO (13 days)</td>
<td>ddI + SAQ (13 days)</td>
</tr>
<tr>
<td>DMSO (8 days), then</td>
<td>SAQ (8 days), then</td>
</tr>
<tr>
<td>DMSO + PBS (13 days)</td>
<td>SAQ + ddI (13 days)</td>
</tr>
</tbody>
</table>

Control treatments (left) are paired with their corresponding drug treatments (right)

3.2.5. Viability

At each subculturing, viability was calculated using a haemocytometer (Hausser Scientific, Horsham, PA, USA). Briefly, 10 μl of cells suspended in medium (after trypsinization) was mixed with an equal volume of trypan blue (Sigma) and pipetted
onto the haemocytometer. The number of cells that were viable (clear) or dead (blue) was counted under a light microscope and the following formula was employed: 
\[
\frac{\text{number of viable cells}}{\text{number of viable cells} + \text{number of dead cells}} \times 100.
\]

### 3.2.6. Population Doubling Time (PDT)

At each subculturing, PDT was calculated using the current number of cells counted in the flask, the initial number of cells placed in the flask and the amount of time (in days) since the flask was seeded with the initial number of cells. The formula used in this calculation is: 
\[
PDT = \frac{\log 2}{\log \frac{N}{N_0}} \times \text{days since seeding},
\]
where \(N\) = final cell number and \(N_0\) = initially seeded cell number.

### 3.2.7. DNA and RNA extractions

DNA and RNA were extracted from cells using the AllPrep DNA/RNA Mini Kit (QIAGEN). Extractions were performed according to the manufacturer's protocol: Procedure for DNA/RNA Extraction of Pelleted Cells. Cells were homogenized using QIAshredder tubes (QIAGEN) and extractions were performed using the QIAcube (QIAGEN). There was one minor change from the above protocol: the DNA was eluted using AE buffer (QIAGEN), rather than the EB buffer provided in the kit.

### 3.2.8. mtDNA quantification

mtDNA was quantified using the same method as the clinical liver biopsy samples, as described in section 2.2.4.
3.2.9. mtRNA quantification

mt-mRNA gene expression was quantified using the same method as the clinical liver biopsy samples, as described in section 2.2.5.

3.2.10. Statistics and data analysis

Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA). All graphs for cell culture comparison were also performed using Excel. The Mann-Whitney statistical test was used to compare cells in the ddI experiment with the following website http://elegans.som.vcu.edu/~leon/stats/utest.html.

3.3 Results

3.3.1. d4T experiment

Treatment of HepG2 cells with d4T in low glucose medium did not alter cell viability or PDT compared to controls, as cell viability was maintained above 92%, whether in high or low glucose (Figure 7). On day 8, there was an artificial decrease in viability to 78% for cells treated with 0.3 and 30 μM d4T in the low glucose medium and an artificial decrease to 68% for cells treated with 0.3 μM d4T in the high glucose medium. The reason for the artificial decreases in viability is that cells were rinsed with basal medium instead of trypsin. When the cells did not enter suspension, cells were scraped off the surface of the flasks and this likely caused an increase in dead cells.
Figure 7: Viability of HepG2 Cells Treated with d4T

On day 8, exposure to 0.3µM and 30µM d4T showed a slight decrease in cell viability in the low glucose medium and 0.3µM d4T showed a decrease in cell viability in the high glucose medium. This experiment was performed once, with each treatment done in duplicate.

Treatment of HepG2 cells with d4T in low glucose medium did not alter PDT compared to controls (Figure 8). All five treatments in the low glucose medium had a PDT close to 1.0 throughout the entire experiment, except for on day 10 when there was a marked increase in PDT to 12.8 and 6.1 in cells treated with 0.3 and 3.0 µM d4T,
respectively. These strange measurements were taken on the same day and appear artefactual; they may have been caused by the cells having been scraped off the flasks. Treatment of HepG2 cells with d4T in high glucose medium also did not alter PDT compared to controls, with values close to 1.5 throughout the experiment (Figure 8). On day 8, there was a marked decrease in PDT to -4.4 and -2.6 in cells treated with 30 and 0.3 µM d4T, respectively. These two measurements were taken on the same day and were likely caused by the cells being scraped off the flasks.
Figure 8: PDT of HepG2 Cells Treated with d4T

On day 10, exposure to 0.3µM and 3µM d4T showed an increase in PDT in the low glucose medium. On day 8, exposure to 0.3µM and 30µM d4T showed a decrease in PDT in the high glucose medium. This experiment was performed once, with each treatment done in duplicate.

mtDNA content and mt-mRNA gene expression were only determined for cells treated with 30 µM d4T and DMSO. Overall, mtDNA content ratios were higher for cells treated with 30 µM d4T compared to DMSO in both the low and high glucose media, although ratios were similar between the two treatments at the end of the experiment.
Increased mt-mRNA gene expression levels were observed in cells treated with 30 µM d4T in both the low and the high glucose medium compared to control treatments with DMSO (Figure 10). There was an increase in mt-mRNA gene expression in the high glucose medium for cells treated with d4T on day 8, likely due to the cells being scraped off the flasks.

*Figure 9: mtDNA/nDNA Ratios of HepG2 Cells Treated with d4T*

MtDNA/nDNA ratios in cells treated with 30µM d4T were higher compared to DMSO-treated control cells, except on day 10 in the high glucose medium where DMSO-treated cells had higher mtDNA/nDNA ratios. This experiment was performed once, with each treatment done in duplicate.
On days 10, 15 and 19, increased mt-mRNA/n-mRNA ratios were observed in cells treated with 30μM d4T in both media. There was a large increase in mt-mRNA/n-mRNA ratios in d4T-treated cells on day 8 in high glucose medium. This experiment was performed once, with each treatment done in duplicate.

3.3.2. ddI experiment

For this experiment, only mtDNA content and mt-mRNA gene expression were considered. Cell viability and PDT were not calculated when cells were subcultured.

Treatment with ddI resulted in decreased mtDNA content (Figure 11) in a time-
dependant manner, in both the low glucose and the high glucose media. However, the medium glucose concentration itself did not influence the mtDNA content of the cell, as the mtDNA/nDNA ratio was similar after 10 days in both control cultures. Average variability between replicates for mtDNA content ratios was 28.8% (Table 16).

**Figure 11: Average mtDNA/nDNA Ratios of HepG2 Cells Treated with ddI**

Treatment with ddI resulted in significantly decreased mtDNA content on day 10, in both the low (n=6) and high (n=6) glucose media.

n = number of replicates, * = significant difference between groups (p<0.05)
### Table 16: ddI Experiment: Variability between Replicates

<table>
<thead>
<tr>
<th>Day</th>
<th>Low glucose + PBS</th>
<th>High glucose + PBS</th>
<th>Low glucose + ddI</th>
<th>High glucose + ddI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>154.5 ± 54.4, 35.2%</td>
<td>115.3 ± 24.9, 21.6%</td>
<td>107.5 ± 19.2, 17.9%</td>
<td>96.6 ± 28.2, 29.1%</td>
</tr>
<tr>
<td>10</td>
<td>164.7 ± 48.1, 29.2%</td>
<td>154.5 ± 54.4, 35.2%</td>
<td>13.6 ± 6.1, 44.5%</td>
<td>10.7 ± 2.7, 25.7%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Low glucose + PBS</th>
<th>High glucose + PBS</th>
<th>Low glucose + ddI</th>
<th>High glucose + ddI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>6.3 ± 2.3, 36.0%</td>
<td>3.5 ± 1.0, 27.6%</td>
<td>5.2 ± 0.9, 16.9%</td>
<td>5.8 ± 3.0, 51.8%</td>
</tr>
<tr>
<td>10</td>
<td>4.1 ± 1.6, 39.8%</td>
<td>4.0 ± 0.8, 20.4%</td>
<td>4.1 ± 1.6, 39.9%</td>
<td>3.2 ± 0.4, 11.6%</td>
</tr>
</tbody>
</table>

SD = standard deviation; %CV = coefficient of variability

Treatment with ddI in low glucose medium did not alter mt-mRNA gene expression (Figure 12). Treatment with ddI in high glucose medium resulted in an initial increase in mt-mRNA gene expression on day 3 that returned to normal on day 10. Average variability between replicates for mt-mRNA gene expression content ratios was 30.5% (Table 16).
Figure 12: Average mt-mRNA Gene Expression Levels of HepG2 Cells Treated with ddl

Treatment of HepG2 cells with ddl in low glucose medium did not alter mt-mRNA gene expression (n=6), while cells treated with ddl in high glucose medium had increased mt-mRNA gene expression on day 3 that returned to normal on day 10 (n=6). n = number of replicates, * = significant difference between groups (p<0.05)

3.3.3. ddl/SAQ experiment

Each flask was initially seeded with 2.0 X 10^6 cells for an initial confluency of approximately 20%. Viability and PDT were only determined on days when flasks were subcultured, since trypsinization was required. Flasks reached 50% confluency 2 days post-seeding and drug and control treatments were added at this time. The flasks treated with 10 X Cmax of SAQ had decreased to 3% confluency 8 days post-treatment and so these treatments, along with their controls, were stopped and repeated with 1 X
Cmax of SAQ and corresponding controls. At this time, four new treatments were initiated with 1 X Cmax of SAQ and corresponding controls.

Treatment of HepG2 cells with ddI and SAQ did not alter cell viability compared to control-treated cells, with cell viability remaining above 95% throughout most of the experiment (Figure 13). Treatment with ddI reduced cell viability to 75% 6 days post-treatment, however, viability returned to normal at the end of the experiment.
Figure 13: Viability of HepG2 Cells Treated with ddI and SAQ

ddi-treated cells showed decreased viability on day 8, 6 days after ddI treatment was initiated. Treatment with SAQ on day 2 did not alter cell viability and it remained unaltered upon addition of ddI on day 10. This experiment was performed once, with each treatment done in duplicate.

HepG2 cell treatment with ddI showed an initial elevation in PDT compared to PBS-treated control cells that returned to near normal levels on day 23 (Figure 14). Cells treated with SAQ had decreased PDT compared to DMSO-treated control cells throughout most of the experiment. Cells co-treated with ddI and SAQ showed
Co-treatment of HepG2 cells with ddI and SAQ showed decreased PDT compared to control-treated cells. This experiment was performed once, with each treatment done in duplicate.

Treatment with ddI caused a decrease in mtDNA content (Figure 15). mtDNA was further decreased upon addition of SAQ. There was an initial rise in mtDNA content for one of the flasks treated with ddI. mtDNA content ratios were not determined for cells treated with SAQ alone or prior to addition of ddI.
Figure 15: mtDNA/nDNA Ratios of HepG2 Cells Treated with ddI and SAQ

ddi was added at day 2 and caused decreased cellular mtDNA/nDNA ratios that were even further decreased upon co-treatment with SAQ that started on day 10. This experiment was performed once, with each treatment done in duplicate.
CHAPTER FOUR: DISCUSSION

4.1 Clinical study

Studying HIV/HCV co-infection in the BC population is particularly relevant due to a higher prevalence compared to the rest of the country. Because we were only enrolling individuals who were undergoing a standard of care biopsy at the request of their physicians, our numbers are relatively low. This was influenced by two things: changes in guidelines pertaining to liver biopsies as well as the demographics of HIV/HCV co-infected individuals in BC. Since the newer guidelines that took effect around 2004 no longer required a liver biopsy for HCV therapy initiation, it is likely that fewer liver biopsies were ordered by physicians. As well, the demographics of the HIV/HCV co-infected population includes many individuals who are either homeless or using illicit drugs, or both, and these individuals are less likely to seek care from a physician and are less likely to be deemed a good candidate for HCV therapy. In an attempt to increase study participant enrolment, enrolment remained open for seven years (from 2003-2010). We were able to enrol 65 study participants and collect 52 double liver biopsies. Thus, lack of enrolment was a major limitation in our study.

There were almost twice as many individuals in the ON HAART group (n=34) compared to in the OFF HAART group (n=18), of which a third were HAART-naïve. There are a number of factors that may explain why the majority of participants were ON HAART and so few were HAART-naïve; one pertains to CD4 count and HAART initiation. Without HAART, CD4 counts will naturally decline to relatively low levels before 10 years post-infection. When this study first began, the recommendations were to initiate HAART in HIV-infected individuals when a CD4 count of 200 cells per µl or less was attained. Therefore, most HIV-infected individuals would have qualified...
for HAART within the first decade of their infection. Part-way through this study in 2009, the recommendation for initiation of HAART was changed to a CD4 count of 350 cells per µl or less, unless co-infected with tuberculosis or hepatitis B virus or if pregnant, in which case HAART was recommended regardless of CD4 count. Therefore, most individuals would have fallen into the ON HAART group and we were particularly limited by study participant numbers in the OFF HAART group. Of note, the newest recommendation in 2012 is to initiate HAART for all HIV-infected individuals, regardless of CD4 count, so that there would arguably be even less people in the OFF HAART group if we were still recruiting for this study today.

The ON and OFF HAART groups had similar CD4 counts, even though HAART improves CD4 counts. This is because people who were not prescribed HAART by their physicians had relatively high CD4 counts and HAART was not essential. This may have introduced a bias since participants in the OFF HAART group had naturally high CD4 counts whereas those in the ON HAART group had naturally lower CD4 counts that necessitated being on HAART to help elevate their CD4 counts. It could be that those in the OFF HAART group had less HIV-related pathology compared to the ON HAART group and this may have prevented us from seeing effects from the HAART drugs.

The gender, age and proportion of HCV genotypes of all study participants combined reflect the characteristics that are observed in the Canadian and BC populations at large. Therefore, our study participant population serves as a good model for both the country and province.

The age of participants in the ON versus OFF HAART groups was similar as was the age of participants on the D-drug versus other NRTIs groups. However, participants in the PI group were, on average, 5 years older than participants in the
NNRTI group. Since age has repeatedly been shown to be an independent variable associated with increased progression of hepatic fibrosis in HCV- and HIV/HCV co-infected individuals, this could have skewed the data that we observed, making it unclear whether differences between groups were due to the different drug regimens or due to differences in age. As well, perhaps we missed significant differences between the two groups due to a significant difference in age.

HAART has been shown to be associated with hepatotoxicity, particularly in the HIV/HCV co-infected population, as evidenced by increased fibrosis progression in patients on HAART versus those off HAART. We therefore expected to see higher IK pathology scores in the ON HAART group compared to the OFF HAART group. However, in agreement with previous results in this cohort, the ON and OFF HAART groups showed similar pathology scores. Although other studies have examined AST platelet ratio index scores, a non-invasive serum marker test for estimating liver fibrosis, as opposed to IK pathology scores, we thought we may see evidence of increased fibrosis via histopathology. Our data may have been influenced by the fact that 13 different medical pathologists scored the biopsies, and so there may be inter-observer variability. Furthermore, since the study was ongoing for seven years, medical pathologists who examined multiple biopsies may have changed the way that they grade and/or stage tissue over the course of the study, also resulting in intra-observer variability.

HIV infection itself is associated with hepatotoxicity and increased hepatic fibrosis so that individuals who were OFF HAART may have been experiencing liver damage from lack of ARV therapy and this may help explain the lack of differences in median IK pathology scores we observed between the two groups. Some groups have found the opposite to our hypothesis; that HAART attenuates liver fibrosis in HIV/HCV
co-infected individuals so that those off HAART would have more fibrosis and hence higher pathology scores, while others have found that type and duration of HAART are predictors of progression of liver fibrosis. These conflicting findings suggest that liver fibrosis progression in these individuals is multifactorial so that competing factors may have confounded our findings with respect to a difference in pathology scores between those on and off HAART. Furthermore, it is possible that by only comparing the total pathology score, rather than comparing each subcategory within the scoring system (i.e. confluent necrosis, portal inflammation, fibrosis, etc.), we may have missed differences between groups since the total score may have masked subcategorial differences. Perhaps we would have seen differences if we had compared each individual category’s score.

PI-containing HAART regimens have been shown to be associated with increased hepatic fibrosis compared to non-PI-containing HAART regimens. However, NNRTI-containing HAART regimens have also been shown to be associated with increased fibrosis. Participants on PIs versus NNRTIs had similar IK pathology scores, perhaps suggesting that both PI- and NNRTI-containing HAART regimens may be associated with similar hepatotoxicity in our cohort.

D-drugs are associated with hepatotoxicity in vitro and D-drug containing HAART regimens have been shown to be more hepatotoxic and lead to increased hepatic fibrosis clinically compared to non-D-drug-containing HAART regimens. We had hypothesized that higher IK pathology scores would be seen in the D-drug group compared to the other NRTIs group. The lack of differences between the groups may be related to inter- and intra-observer variability, as well as the small number of participants who were taking a D-drug since they are no longer commonly prescribed in Canada.
NRTIs, PIs and NNRTIs have all been shown to be associated with mitochondrial alterations, affecting levels of mtDNA in hepatocytes and other cells. Despite this, the ON HAART group showed similar mtDNA levels as the OFF HAART group. These findings support previously published data of a lack of differences in mtDNA levels between the two groups. A larger cohort of patients may be necessary to detect subtle differences in mtDNA levels between the two groups. HIV itself is also associated with mtDNA depletion and this may confound our findings. The variability in HAART regimens may also confound our findings when comparing these two groups.

As D-drugs are clearly associated with depleted hepatic mtDNA quantity, we hypothesized that we would see altered mtDNA quantity in the D-drug group compared to the other NRTI group. Again, the small sample size, particularly in the D-drug group, likely limited our ability to address this.

Although mt-mRNA levels have been shown to be affected by certain HAART drugs, they have not been extensively studied. Being ON or OFF HAART did not appear to affect mt-mRNA levels nor did being on a D-drug. However, being on an NNRTI-containing regimen may be associated with lower mt-mRNA compared to being on a PI. The small amount of sample available for mRNA studies limits the strength of these findings, nevertheless, it may be that the NNRTIs were causing hepatotoxicity, as others have shown, and this resulted in decreased mt-mRNA expression levels. Although the mechanism of NNRTI-related hepatotoxicity is unknown, it could be related to hypersensitivity reactions, since these have been associated with NNRTIs. Alternatively, it could be that the NNRTIs were interacting with CYP enzymes in the liver leading to decreased hepatic function that then resulted in decreased hepatic mt-mRNA levels. Another possibility is that individuals on NNRTIs had normal mt-mRNA levels and individuals on PIs had elevated levels. PIs are associated with
elevated liver enzymes, an indication of hepatic inflammation that may be related to hepatic function \textsuperscript{62, 63}, so that perhaps the livers of people on PIs are trying to compensate for loss in hepatic function by boosting hepatic mt-mRNA levels. Because we did not have access to liver tissue from healthy uninfected controls, we cannot distinguish between these possibilities. PIs are also associated with drug interactions due to their disruption of CYP 3A4 activity that can result in higher concentrations of other drugs \textsuperscript{64-66} so that perhaps these other drugs caused increased mt-mRNA levels in the liver.

Several authors have reported NRTI-related increases in mtDNA mutations in HIV-infected individuals \textsuperscript{60, 208, 233}. However, little is known about mtDNA deletions in the context of HAART, whether in HIV-infected or HIV/HCV co-infected populations. We saw no significant differences in median percent mtDNA deletions between the PI and the NNRTI groups or between the D-drug and the non-D-drug groups. The lack of differences may have been, at least in part, due to the small number of participants in these groups. To our knowledge, it is unknown whether PIs or NNRTIs have an effect on mtDNA quality. Indeed, PIs and NNRTIs are not believed to invoke mtDNA mutations and deletions via the mechanism of NRTI-related inhibitions of polymerase-gamma \textsuperscript{59, 60, 208}. It could be that PIs and NNRTIs do not promote changes in mtDNA quality or it could be that both drug groups cause similar changes in mtDNA quality so that there are no differences between the groups.

MDR1 transports drugs out of cells \textsuperscript{235} and so it may play a role in hepatic drug toxicity, although, to our knowledge, its activity has never before been examined in the HIV/HCV co-infected population. Certain HAART drugs have been shown to interact with MDR1 activity \textsuperscript{236}. However, none of the factors studied (ON vs. OFF HAART, PI vs. NNRTI) showed any association with MDR1 gene expression. This may have been
due to competing inhibition of MDR1 activity by NRTIs \(^{238}\) and induction of MDR1 activity by PIs and NNRTIs \(^{239}\). There were too few subjects on D-drugs with MDR1 mRNA results (N=2) to compare based on this characteristic.

Higher rates of liver-related morbidities and mortalities are observed in the HIV/HCV co-infected population compared to HIV- or HCV-mono infected individuals \(^{101, 190}\) and this may be affected by HAART drugs that are metabolized by the liver. We failed to show an association between HAART and differences in mtDNA quantity or quality or in mt-mRNA levels, and we failed to explain differences in toxicity between the groups by a lack of differences in MDR1 activity between same groups.

4.2 Cell culture studies

Treatment of HepG2 cells with d4T has been shown by others to result in a dose- and time-dependent decrease in cell number as well as mtDNA quantity \(^{56, 60, 227, 242}\). Some have reported increases in cell growth and mtDNA quantity in the first 5-15 days of culture, depending on the concentration of d4T exposure \(^{56, 227}\). Our results suggest that there was a problem with our experiment, since we did not observe any consistent changes in mtDNA or cell growth, something that has been well documented by others in the same cell line. Perhaps there was a problem with some of the subculturing techniques that were employed (pipetting, mixing, trypsinisation and/or splitting cells), since this was my first experience with cell culture. When we later checked the reproducibility of my techniques, it became clear that I needed to improve these techniques before moving on to another experiment.

To our knowledge, examination of alterations in mt-mRNA gene expression in HepG2 cells exposed to d4T has never been studied. We observed increased mt-mRNA gene expression in cells treated with 30 \(\mu\)M d4T in both low and high glucose
media compared to control treatments with DMSO. It is, however, impossible to say whether this increase is due to d4T treatment or poor cell culture technique. Since treatment with d4T in both low and high glucose media consistently showed increased mt-mRNA gene expression throughout the experiment, the increased expression observed may indeed be from d4T exposure.

The level of glucose in the medium did not appear to influence cell viability, PDT, mtDNA quantity or mt-mRNA gene expression of d4T-treated cells. This may be because the difference in media glucose concentration has no effect on these parameters. Low glucose was used to reduce the amount of glucose available for glycolysis thus inducing the cells to use OXPHOS. We rationalized that if OXPHOS was required, it may induce mitochondrial biogenesis that would result in increased mtDNA replication and may amplify the toxic effects of the drugs, if any. However, based on our experiments, since there was no difference in mtDNA content of cells cultured under both conditions, we have no evidence that OXPHOS was induced by the low glucose used here. Of course, it may be because there were problems with my technique during this experiment.

Treatment with ddI resulted in decreased mtDNA content in a time-dependant manner, in agreement with previously published data 56, 60. Despite an average variability between replicates of 28.8%, the dramatic decline in mtDNA quantity after 10 days of culture in the presence of 10 X Cmax ddI was convincing evidence of ddI-induced mitochondrial toxicity. The glucose concentration did not amplify this result, once again suggesting that growing HepG2 cells in low glucose medium did not increase effects from mitochondrial toxicity from ddI. The difference in glucose concentrations may need to be larger to see an effect or we may have seen greater differences if we had also altered the amount of glutamine present in the media, since
glutamine is used by cancer cells when glucose is not readily available \(^{272}\). Normal glutamine levels in the low glucose medium may have, therefore, prevented the desired induction of OXPHOS.

The effects of ddI on mt-mRNA gene expression in HepG2 were studied in low and normal glucose media concentrations. We saw a completely different pattern in mt-mRNA gene expression changes from ddI in the two media. This suggests that the level of glucose in the medium used plays a role in modulating mitochondrial mt-mRNA levels in the presence of ddI. Perhaps cells grown in the low glucose medium were better able to adapt to ddI toxicity and that is why levels were similar to controls on both days. The initial rise in mt-mRNA levels observed in the high glucose medium that was then followed by a return to normal mt-mRNA levels suggests that perhaps cells were overcompensating to the mitochondrial toxicity on day 3 and then were able to adapt to a steady-state by day 10. It is unkown why these changes occurred as they did since a mechanism was not investigated.

To our knowledge, SAQ has never before been examined for hepatic mitochondrial toxicity in HepG2 cells. We observed almost complete cell death from treatment of cells with 10 X Cmax of SAQ, eight days post-treatment, which was surprising since 10 X Cmax of ddI did not cause this amount of cell death and ddI is known to be highly toxic to hepatocytes \(^{56, 227, 241}\). Treatment of cells with 10 X Cmax of ddI caused cell death, as previously reported by others \(^{56, 241}\), however, we observed cell viability return to normal within 17 days, unlike these authors. The reason for this discrepancy is likely that we were using low glucose medium, whereas these authors were using normal glucose medium (1,000 vs. 4,500 mg/L). This suggests that cells are better able to adapt to ddI toxicity-related cell death in the presence of low glucose compared to normal glucose. Glucose deprivation may have induced a metabolic shift
in the surviving cells that allowed them to better tolerate ddl toxicity. A similar phenomenon was demonstrated by a group who showed that human hepatoma cells that were deprived of glucose were able to induce autophagy to eliminate damaged proteins, thus enabling the cells to better withstand cell death stimuli. When ddl was added to cells that had already been treated with 1 X Cmax of SAQ for eight days, there was no alteration in cell viability, suggesting that SAQ protected the cells from cell death that would normally be caused by ddl.

An initial elevation in PDT was observed in HepG2 cells treated with ddl compared to control-treated cells. This suggests that the cells were responding to ddl toxicity by increasing cell number, however, they were overcompensating and PDT was increased. PDT slowly returned to normal by day 23 for ddl-treated cells suggesting that the cells were eventually able to better tolerate ddl-related toxicity with respect to PDT. Decreased PDT was observed in cells treated with both ddl and SAQ and PDT was further decreased upon co-treatment with the two drugs. This suggests that ddl and SAQ act together to potentiate toxicity-related PDT depletion. Arguably, if the cells had been treated with the drugs for longer, we may have seen the same type of metabolic shift effect that was observed when cell viability returned to normal.

Decreased mtDNA quantity was observed in HepG2 cells treated with ddl and mtDNA quantity was further decreased upon addition of SAQ. This suggests that ddl and SAQ cause complementary hepatic mitochondrial toxicity in these cells. It is unknown if the same synergy would have been observed if the cells had been treated first with SAQ and then with ddl, since this was not done.
4.3 Future directions

As mentioned, the clinical study was limited by small sample size. In the future, the clinical study could be repeated with a larger sample size. When comparing the outcomes of interest, it would also be important that the groups be well equilibrated with respect to age, since this can be an important confounder. Hepatic mtDNA deletions\(^{274}\) and mutations\(^{275}\) have been shown to be age-dependent so that when studying these changes in a cohort, the groups must be similar in age. An investigation using mtDNA sequencing could be conducted, in order to determine what positions of the mtDNA genome are affected by mutations or deletions. As well, with respect to IK pathology scores, subcategories within the scoring system could be compared in the future to ensure that differences between groups are not missed due to only looking at the total score.

The original grant sought to investigate differences in pre- versus post-HCV therapy biopsies, however, given the time constraints, we only investigated pre-HCV therapy biopsies. Therefore, in the future, post-HCV therapy biopsies could be examined for differences in mtDNA quantity and quality, mtRNA quantity and MDR1 activity between ON and OFF HAART groups, PI- versus NNRTI-containing HAART regimens and D-drugs versus other NRTIs. Additionally, a patient’s own pre- versus post-HCV therapy biopsy could be compared in order to investigate whether HCV therapy plays a role in liver mitochondrial toxicity. Investigating whether some of the factors studied are associated with differences in HCV therapy outcome (i.e. SVR, transient- or non-response) may also be of interest. In addition to double-liver biopsies, mouth swabs and blood samples were collected from study participants before during and after HCV therapy, so these samples could be compared for alterations in mtDNA quantity and quality, mtRNA quantity and MDR1 activity.
In addition to the investigations that we made here, the mechanisms underlying changes in mtDNA and mtRNA quantity that have been observed by us and others could be investigated. Mitophagy and/or mitochondrial biogenesis (Figure 16) may play a role in HAART-related mitochondrial toxicity, since mtDNA and mtRNA levels do not always increase or decrease in unison \(^{229}\), as you might expect. To explain this, perhaps increased mtDNA levels are accompanied by decreased mtRNA levels because the cells adapt to mitochondrial toxicity by increasing mitochondrial biogenesis. Another possible explanation is that HAART may inhibit autophagy so that damaged mitochondria are not properly degraded. The mtDNA from damaged mitochondria would likely be unable to transcribe mt-mRNA so that mtDNA levels would be higher than mtRNA levels. An investigation into mRNA levels, as well as protein levels (since protein was also extracted from study participant liver samples), of genes involved in the various stages of mitophagy and mitochondrial biogenesis may shed light on whether this mechanism is involved in HAART-related mitochondrial toxicity. Our lab is currently investigating this using the liver RNA from this cohort of study participants.
In the future, the d4T experiment could be repeated with proper cell culture technique in order to examine whether there are differences in HepG2 cell viability, PDT and/or mtDNA quantity between cells in low versus high glucose medium. If there are differences, then this might suggest that researchers could use the medium that shows greater response to mitochondrial stress so that small effects from different HAART drugs are not missed. Media containing low glutamine levels in addition to low glucose levels could also be examined to see if HAART drug toxicity is more apparent. Several groups have shown that HepG2 cells grown in high galactose and low glucose containing medium use more OXPHOS and are more susceptible to mitochondrial insults from toxins $^{278, 279}$, so in the future this type of medium would also be important to compare. Any effect seen should be ideally studied to demonstrate dose and time.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure16.png}
\caption{Mitochondrial Biogenesis and Mitochondrial Autophagy (Mitophagy)}
\end{figure}

A) Mitochondrial biogenesis is the cellular production of new mitochondria. A mitochondrion grows in size and then segregates into two daughter mitochondria. B) Mitophagy is the selective autophagy of mitochondria. Damaged mitochondria are sequestered by an autophagosome that fuses with a lysosome for hydrolytic lysosomal degradation of the mitochondria.

Ref $^{276, 277}$
dependency. Once mitochondrial differences are observed, then an investigation into the mechanism of these changes could be performed.

In the future, the ddI/SAQ experiment could be repeated and carried out for a longer period of time to determine if there would be any further alterations in HepG2 cell viability, PDT and/or mtDNA quantity or if levels would eventually return to normal. As well, alterations in mt-mRNA gene expression levels could be examined. Finally, attempting to identify a mechanism for all the alterations that are seen could be of interest.

In the future, HepG2 cells could be treated with various different HAART drugs from all of the different classes of HAART drugs, alone and in combination, over the period of several months and investigate longitudinal alterations in mtDNA quantity and mt-mRNA gene expression levels. This could be done in both low and high glucose media and in medium containing both low glucose and low glutamine or high galactose in order to determine whether one is more responsive to the mitochondrial stress that may be caused by the various HAART drugs. HepG2 cells could be treated with different HAART drugs in combination with the antiviral drugs used in HCV therapy in order to examine the effects of HCV therapy on HAART-related hepatic mitochondrial toxicity.

In the future, it may be interesting to investigate HAART drugs and HCV drugs in HIV and/or HCV infected cells. This would allow the investigation of drug-related toxicity in the context of viral effects. While HepG2 cells support HIV infection$^{280}$, they do not support HCV infection$^{281}$. The human hepatocellular carcinoma cell line Huh-7.5.1 supports HIV/HCV co-infection$^{282}$ so that in the future these cells could be exposed to different combinations of HAART drugs in addition to drugs used in HCV therapy to examine hepatic mitochondrial toxicity. However, one of the reasons for
using HepG2 cells in our experiments was that these cells are known to be capable of triphosphorylating NRTIs into their active form, a step required to test NRTI activity and toxicity in cells. Thus, it would first have to be determined whether Huh-7.5.1 cells are capable of triphosphorylating NRTIs prior to conducting HAART toxicity-related experiments in the cells. Huh-7.5.1 cells are capable of triphosphorylating ribavirin into its active form so it is possible that these cells would also be capable of phosphorylating NRTIs, making them a good model for these experiments in the future.

4.4 Conclusion

Treatment with HAART has drastically decreased mortality and increased morbidity in persons infected with HIV, in addition to lowering the risk of viral transmission. However, toxicity, including hepatic mitochondrial toxicity, may be a concern for individuals on HAART. With increased HAART-related toxicity and more severe disease progression in HIV/HCV co-infected individuals, hepatic mitochondrial toxicity is an even greater concern. A better understanding of the effects of HAART on the livers of persons co-infected with HIV and HCV could affect millions of people worldwide and possibly influence therapy guidelines and outcomes. Although HIV/HCV co-infected individuals currently have decreased HCV therapy success, with multiple new direct acting antiviral drugs for HCV therapy on the horizon, they are likely to have better SVR rates in the future.

The first goal of this study was to investigate hepatic mitochondrial toxicity in HIV/HCV co-infected individuals and compare patients were ON versus OFF HAART and patients who were on PIs versus NNRTIs and on D-drugs versus other NRTIs. Although the clinical study failed to show significant alterations in mtDNA quantity or quality or MDR1 gene expression between groups, we showed that there may be a
difference in mt-mRNA gene expression between the PI and the NNRTI group. The second goal of this study was to investigate hepatic mitochondrial toxicity from HAART in HepG2 cells. For the first time, we showed that glucose levels in the media may alter ddI-treated HepG2 cell mtDNA quantity and mt-mRNA gene expression levels. We also showed for the first time that SAQ reduces HepG2 cell PDT and mtDNA quantity and ddI further reduces these measurements when the two drugs are co-administered.

Further investigation into HIV and HCV therapy-related hepatic mitochondrial toxicity is warranted to gain a better understanding of the effects of these drugs on hepatic mitochondria.
References


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Appendix

Patient Informed Consent Form
PATIENT INFORMED CONSENT

Mitochondrial toxicity in HIV/HCV coinfection antiviral therapy.

(Study of Drug related mitochondrial toxicity in HIV and HCV antiretroviral therapy: impact of mitochondrial DNA/nuclear DNA ratio changes on therapy outcome)

Principal Investigators: Dr. Valentina Montessori (604) 806-8644
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Co-investigators: Dr. Marianne Harris (604) 806-8771
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Dr. Rolando Barrios (604) 806-9296
Dr. John Farley (604) 687-1147
Dr. Alnoor Ramji (604) 688-6332 ext 225
Dr. Robert Enns (604) 688-6332 ext 222
Dr. David Walker (604) 806-8346 ext 62705

24 hr. contact: (604) 682-2344 ask for Infectious Disease Doctor on call

Study sponsors: CIHR and MSFHR

You should know that Drs. Côté and Montaner are inventors on a patent that has been filed by the University of British Columbia on the test used in this study. Therefore, they and UBC could one day derive a financial benefit from this research.

You are invited to participate in this study because you are infected with both the human immunodeficiency virus (HIV) and the Hepatitis C virus (HCV) and are about to start an antiviral regimen. Your doctor has already ordered a liver biopsy (small piece of your liver is collected using a needle) to be done and prescribed an antiretroviral regimen for you.

Before you agree to participate you should be aware of the following:
iv) Your participation in this study is entirely voluntary (by choice).
v) Before your decide whether or not to take part in this study, the purpose of the study, how it
may help others or yourself, risks of the study and what is expected of you will be explained
to you.
vi) This consent form gives you information about the study.
vii) The study will also be explained to you.
viii) Ask questions at any time that will help you to understand the study so that you feel fully
informed.
ix) You may decide not to take part or to withdraw from the study at any time without affecting
your routine medical care.
x) Once you understand the study, and if you decide to take part, you will be asked to sign this
consent form and you will be given a copy to keep.

Purpose of this Study
The purpose of this research is to study your blood and liver for potential signs of mitochondrial
toxicity that can be caused by the antiviral drug(s) used to treat your disease. Mitochondria are
present in all cells and are responsible for most of the body’s energy production. Some of the
drugs used in antiretroviral therapy for HIV or HCV can alter the amount of DNA contained in
the mitochondria (mitochondrial DNA), which can cause mitochondrial dysfunction and lead to
a variety of drug-related adverse effects. The purpose of this study is to measure levels of
mitochondrial DNA and mitochondrial RNA. Mitochondrial RNA is a messenger that acts as an
intermediate during the production of mitochondrial proteins. Mitochondrial proteins are the
molecules involved in generating the body’s energy. These measurements will be done in blood,
mouth swab and liver samples, and we will determine whether there is a relationship between
these measures and the outcome of your antiviral therapy, as well as the occurrence of therapy-
related adverse effects. We will also examine your liver mitochondria under an electronic
microscope.

The sponsors of this study are the Canadian Institute for Health Research (CIHR) and the
Michael Smith Foundation for Health Research (MSFHR).

Study Procedures
You will be asked to donate one blood sample (1-2 tablespoon) at each of your visit to the
doctor throughout your therapy, for up to 48 weeks. This should represent between 5 and 8
blood samples total. One sample will be collected before you initiate antiretroviral therapy,
another one after one month, and at every visit to your doctor after that, until the end of your
therapy or 48 weeks, whichever comes first. This blood sampling will be performed at the St.
Paul’s Hospital laboratory. Every effort will be made to collect this sample when you are having
other blood work done so that it does not require any extra time on your part. We will also ask
you, on the same days you’re giving blood, to rub a swab on the inside of your mouth.

As part of standard care, patients such as yourself usually have a liver biopsy (small piece of
your liver is collected by inserting a needle through your abdomen, under local anesthe'sia) done
before starting treatment, to evaluate the state of the liver. We will be collecting a little more
tissue than usual during this procedure, to have enough for the pathologist and for our study.
Also, at the end of your therapy or if you interrupt treatment because of therapy-related adverse
effects, a second liver biopsy (also collecting a little more tissue than normal) will be performed.
For this study, procedures above the standard of care are that during your first biopsy, we will collect a little more liver tissue and that a second biopsy will be done. The time required for this extra procedure is approximately half a day.

**Inclusion and exclusion criteria**

To take part in this study you must:
1. be HIV and HCV positive
2. not be currently taking HCV antivirals nor having taken any in the past. However, you can be receiving HIV antiretrovirals (antiretrovirals are the medications used to control HIV and AIDS) and if you have been taking them for more than 6 months
3. be 19 years of age or older
4. be able and willing to give informed consent

You cannot take part in this study if you:
1. are pregnant or breastfeeding
2. are a woman of childbearing age and you are not willing to avoid becoming pregnant during the study period
3. are coinfected with hepatitis B virus, have metabolic liver disease or currently suffer from opportunistic infections (AIDS).
4. Are currently receiving the HIV drug didanosine (ddI or Videx)

**Risks Involved with the Study:**

**Blood draw:** risks associated with drawing blood include minor discomfort, soreness at the level of needle entry, bleeding under the skin (hematoma), rarely infection, and fainting. All efforts will be made to avoid these risks.

**Liver biopsy:** risks and complications associated with small core needle liver biopsy are pain (up to 62% immediately following the procedure) that quickly subsides in most cases (80% pain-free after 24 hours). Death is also a rare risk of the procedure (between 0.018% and 0.031%, or up to 1 in 3200 biopsies), usually caused by bleeding. The fact that two small samples will be collected during each biopsy may at most double these risks. However, it should be noted that the biopsies will be done using newer and safer procedures (ultrasound image-guided using a spring-loaded biopsy gun) which will diminish the risks compared to the numbers stated above. A recent but small study (N=250 persons) using these more recent procedures reported 1.6% minor complications (pain, bruising) and no major complications or death.

**Potential Benefits**

There are no benefits associated with taking part in this study. However, knowledge concerning the effects of antiretroviral therapy on blood, mouth cells and liver mitochondrial DNA in HIV/HCV coinfected individuals may provide insight into what is the best way to treat patients such as yourself in the future.

There is no monetary payment for participating in this study. However, on the day of your second biopsy, we will compensate you for parking and meal expenses ($50).

**Confidentiality**
Your confidentiality will be respected. Information that discloses your identity will not be released without your consent unless required by law or regulation. However, research records and medical records identifying you may be inspected in the presence of the investigator or her designate, by representatives of the Health Protection Branch (HPB) and the UBC/PHC Research Ethics Board for the purposes of monitoring the research. No records that identify you by name or initials will be allowed to leave the investigator’s office. If the results of this study were to be published in a medical journal, your confidentiality would be preserved by identifying you only by a code number.

**Participant Consent**
By signing this consent form, you agree to donate between 5 and 8 small specimen of blood (approximately 2 tablespoons). Every effort will be made to take these blood samples during blood tests already prescribed by your physician. You also agree to undergo an elective liver biopsy when requested by your doctor. Upon completion of this project, your samples will be discarded once all the study assays and analyses are completed. You also consent to the investigators accessing relevant laboratory test results such as plasma viral load tests, liver function tests, blood tests. Any finding in this study that may be relevant to your health will be discussed with you immediately.

By signing this form, you in no way give up any of your rights and you do not release the study doctors or other participating institutions from their legal and professional responsibilities. Your participation is entirely voluntary. You have the right to refuse to participate in this study. If you decide to participate, your decision is not binding and you may choose to withdraw from the study at any time, at which time all of your stored samples will be discarded. Your refusal to participate or withdraw from the study will not have any negative consequences to the medical care, education, or other services you may receive from this clinic or this hospital. 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’ at 604-822-8598 or the Chair of the UBC/PHC Research Ethics Board at 604-682-2344 ext 62325.

I have read the above and have had all my questions about this study satisfactorily answered by one of the principal investigators or their designate and I have received a copy of this consent form for my files.

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