Ultrastructural Changes and Mitochondrial DNA Content in the Hepatocytes of Individuals Co-Infected with HIV and Hepatitis C Virus Following HCV Combination Therapy

by

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Abstract

**Background and objectives:** Hepatitis C Virus (HCV) affects approximately 170 million people worldwide. Some estimates suggest up to 30% of these individuals are also infected with Human Immunodeficiency Virus (HIV). In Vancouver, British Columbia, the percent of HIV/HCV co-infected individuals is even greater, approaching 50% of those infected with HIV. Since the advent of highly active anti-retroviral therapy (HAART), the leading cause of death for HIV positive individuals is end stage liver disease often associated with chronic HCV infection. Previous work by our laboratory did not provide evidence of significant hepatocellular or mitochondrial toxicity associated with HAART treatment. Since it is suggested that drugs used in both HAART and HCV combination therapy may be toxic to the liver, the concurrent use of these treatments in co-infected individuals may be associated with increased hepatocellular and mitochondrial toxicity. The objective of this study was to determine the ultrastructural and mitochondrial DNA content changes that occur in liver hepatocytes in response to HCV combination therapy in co-infected individuals.

**Hypothesis:** HCV combination therapy is associated with ultrastructural hepatocellular changes consistent with either damage or tissue repair in HIV and HCV co-infected patients.

**Methods:** Liver biopsies were taken from co-infected patients before and after receiving HCV combination therapy. One biopsy was used for pathology assessment, the other was divided for nucleic acid assays and transmission electron microscopy (TEM). TEM samples were then examined for ultrastructural changes in hepatocytes following HCV combination therapy.
**Results:** Biopsies from 11 subjects were collected. Of the subjects examined, liver pathology improved significantly following HCV combination therapy while no differences were observed in the mtDNA to nDNA ratio before and after treatment. Ultrastructural analysis of hepatocytes revealed a significant decrease in lipid content following HCV combination therapy.

**Conclusions:** Overall, these results suggest that HCV combination therapy is beneficial to liver health and lessens liver pathology in HIV/HCV co-infected patients featuring a reduction in lipid content or steatosis. Future studies involving greater sample size are required to determine additional effects of HCV combination therapy on co-infected individuals.
Preface

Ethics approval for the clinical study was issued by the University of British Columbia – Providence Health Care Research Ethics Board (UBC-PHC REB), certificate # H03-50055.
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List of Acronyms and Abbreviations

AIDS: Acquired Immunodeficiency Syndrome
ALT: Alanine Transferase
AP: Alkaline Phosphatase
ART: Antiretroviral Therapy
ARV: Antiretroviral (drug)
ASPG: Accessory Subunit of Polymerase Gamma
AST: Aspartate Transferase
AZT: Azidothymidine
BC-CfE: BC Centre for Excellence in HIV/AIDS
CCOI: Cytochrome C Oxidase subunit I
ddC: Zalcitabine
ddi: Didanosine
d-drugs: Dideoxy-NRTIs
d4T: Stavudine
DNA: Deoxyribonucleic Acid
DTES: Downtown Eastside (*impoverished inner-city neighbourhood of Vancouver*)
Epon: Eponate (epoxy resin embedding medium)
ER: Endoplasmic Reticulum
ESLD: End-Stage Liver Disease
FDA: US Food and Drug Administration
HAART: Highly Active Anti-Retroviral Therapy
HCC: Hepatocellular Carcinoma
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
IDU: Intravenous Drug Use
IK-HAI: Ishak-Knodell Histological Activity Index
LDL: Low Density Lipoprotein
mtDNA: Mitochondrial DNA
MSM: Men who have Sex with Men
nDNA: Nuclear DNA
NRTI: Nucleoside/Nucleotide (analogue) Reverse Transcriptase Inhibitor
NNRTI: Non-Nucleoside (analogue) Reverse Transcriptase Inhibitor
PCP: Pneumocystis Pneumonia
PEG-IFN\(\alpha\): PEGylated Interferon alpha \(\text{(PEG: polyethylene glycol)}\)
PI: Protease Inhibitor
RBV: Ribavirin
RNA: Ribonucleic Acid
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
SIV: Simian Immunodeficiency Virus
SNP: Single Nucleotide Polymorphism
SVR: Sustained Virologic Response
TB: Tuberculosis
TEM: Transmission Electron Microscopy
UNAIDS: Joint United Nations Programme on HIV/AIDS
VLDL: Very Low Density Lipoprotein
WHO: World Health Organization
ZDV: Zidovudine
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1. Introduction

1.1 Human Immunodeficiency Virus (HIV)

1.1.1 HIV Epidemiology

The world’s first cases of acquired immunodeficiency syndrome (AIDS) are thought to have originated in south-eastern Cameroon, when a strain of primate virus, simian immunodeficiency virus, crossed the species gap giving rise to HIV (human immunodeficiency virus) early in the twentieth century [1, 2].

In the early 1980s, an outbreak of what would be discovered to be HIV occurred among homosexual males in North America. The AIDS pandemic would continue to spread around the world largely by sexual contact (primarily heterosexual), to date causing the deaths of an estimated 30 million individuals from AIDS-related illness [2-4]. In 2010, there were an estimated 34 million people worldwide living with HIV [4]. Though these numbers are dire, the number of new HIV infections per annum worldwide has begun to steadily decline as there were 21% fewer new HIV infections in 2010 (2.5 million) than in 1997) [4]. According to the same report, 0.8% of the adult global population is HIV-positive, with 5% prevalence among adults in the sub-Saharan African region. Closer to home, North America has an estimated 0.6% prevalence of HIV infections in adults [4].

In Canada, the HIV burden is lower than that worldwide; an estimated 0.3% of adults Canadians were thought to be infected in 2008 and 2009 [5, 6]. Demographic changes are evident over the years; in Canada between 1981 and 1986, an estimated 87% of HIV-positive individuals were men who had sex with men (MSM) [7, 8]. By 2011, MSM comprised less than
half (46.7%) of the known exposure routes in HIV-positive individuals in [9]. Figure 1 shows the known routes of transmission of reported HIV infections in Canada in 2011. According to the Public Health Agency of Canada, among the cases of HIV transmission for which the route of exposure was known, the top three transmission sources were sexual activity among MSM, heterosexual contact and intravenous drug use (IDU) [10]. Here heterosexual transmission includes both endemic (16.4%) and non-endemic (20.8%) heterosexual individuals.

![Figure 1. Reported HIV Infections in Canada in 2011 by Route of Exposure.](Adapted from data by Public Health Agency of Canada. *Estimates of HIV Prevalence and Incidence in Canada, 2011.*).
Despite decreases in the number of new HIV infections in Canada in the years since 2008 [9], Aboriginal populations are overrepresented in the HIV/AIDS epidemic in Canada [11]. A 2007 pilot study done on behalf of the Assembly of First Nations and aimed at studying HIV education in First Nations youth revealed a number of misconceptions and potential risk factors [12]. For examples, surveys indicated that 50% of respondents were not aware that HIV could be transmitted via tattooing or body piercing. The same percentage believed that use of the birth control pill, diaphragm or spermicidal jelly was sufficient to protect against HIV infection. A fifth of respondents (20%) were unaware that IDU was a risk factor for transmission of HIV, and finally, 20% did not want to know if their sexual partner was HIV-positive. These statistics are especially alarming as Aboriginal youth between the ages of 15 to 29 have a greater rate of HIV infection compared to the general Canadian population at large and the rates of HIV amongst Aboriginal groups in Canada have been steadily increasing [11-13]. In British Columbia in 2009, 16.6% of all new HIV infections were diagnosed among Aboriginal people, despite the latter comprising 5% of the population [14].

According to the British Columbia Centre for Excellence in HIV/AIDS (BC-CfE), the “vast majority” of HIV-positive British Columbians reside within the Vancouver Coastal Health Authority catchment [15]. In the City of Vancouver, the rate of HIV infections decreased from 27.8 per 100 000 in 2008 to 23.6 per 100 000 in 2009 [14]. A 2012 study by Milloy et al. based out of Vancouver, examined the health status of people living with HIV/AIDS who are homeless or have marginal housing accessibility in urban centres such as ours [16]. Their findings indicated that “inferior housing status” is a common circumstance for people living with HIV/AIDS, and is strongly correlated to poor treatment outcomes, poor access to- and poor compliance with antiretroviral treatment. This is of particular relevance to our study population,
as many of the participants are geographically located within the downtown east-side (DTES) of Vancouver, some with no permanent address. Further, the BC-CfE reports that individuals residing in the DTES have the lowest life expectancy in Canada, and an HIV seropositivity incidence of 27% amongst intravenous drug users, the highest in the Western world [15]. As a result, initiatives such as the ‘treatment as prevention’ STOP HIV/AIDS research programme championed by Dr Julio Montaner (BC-CfE) [17] aiming to provide improved access to Highly Active Antiretroviral Therapy (HAART), and the controversial InSite supervised injection site (Vancouver Coastal Health Authority) the first of its kind in North America, [18, 19] were initiated as new and innovative strategies for combating the spread of HIV in Vancouver. In 2012, Dr Julio Montaner also reported that British Columbia was the only province in Canada to see “a significant decrease in the number of new HIV infections” and prevention programmes such as STOP HIV/AIDS are being credited [20, 21].

1.1.2 HIV Pathology

The HIV virus belongs to the Retroviridae family, in the genus Lentiviridae. It is an enveloped virus of approximately 120 nm in diameter containing two copies of its genome. The HIV genome consists of single-stranded, positive-sense RNA encoding for a viral protease, reverse transcriptase, integrase and other proteins such as capsid proteins (Figure 2). There are two strains of HIV; HIV-1 is responsible for the worldwide HIV/AIDS pandemic, whereas HIV-2 is confined almost exclusively to West Africa. Both viruses are believed to have jumped the species barrier from primates to humans, and have differing presumed sources; HIV-1 is thought to have originated from a strain of simian immunodeficiency virus (SIV) in chimpanzees, and
HIV-2 from a different strain of SIV found in sooty mangabeys [22]. The use of the acronym “HIV” in this document refers to HIV-1.

**Figure 2. Artistic Representation of a Cross-Sectional View of the Mature HIV Virion.**

HIV may be present in the following bodily secretions: blood (and blood-derived products), semen and pre-seminal fluids, vaginal secretions, breast milk, and possibly saliva, cerebrospinal fluid, amniotic fluid and synovial fluid [23, 24]. A study comparing the relative viral loads in bodily fluids noted that in non-blood fluids the viral loads were 1 to 2 logs lower than those from plasma in the corresponding individuals [23].
Entry of the virus into host immune cells (Figure 3) is mediated by the viral envelope glycoprotein 120 (gp120) and by the cluster of differentiation 4 (CD4) receptor found on T-lymphocytes (helper T cells), macrophages, monocytes and dendritic cells. Binding of gp120 to CD4 results in a conformational change allowing the binding of gp120 with an additional host cell receptor – the chemokine receptors CCR5 (also known as CD195) or CXCR4 (also known as CD184) [25]. A complex is then formed with the viral protein gp41, which in turn mediates a conformational change allowing for the fusion of the viral and cellular membranes [26]. The cytoplasm of the host cell is thereby accessible to the viral core of HIV.

Following entry into the cytoplasm, the viral reverse transcriptase is activated and the single-stranded RNA viral genome is transcribed into complementary double-stranded DNA (cDNA) [27]. The cDNA enters the host cell nucleoplasm and is incorporated into the host genome at one of many possible locations via the actions of viral integrase [28].

After integration of the viral dsDNA into the host genome, latency or immediate transcription and translation of the inserted viral DNA can occur. A current theory on the latency of HIV involves its integration into a region of closed heterochromatin as opposed to transcriptionally active euchromatin [29]. However, it should be noted that HIV encodes for its own transcriptional factor, Tat protein, which can drastically improve the efficiency of viral transcription [30]. Latency in HIV infections is problematic; complete eradication of the virus from the infected host is compromised as latent cells with integrated viral DNA currently cannot be targeted.
2. Co-receptor CCR5 and CD4 mediate fusion and cell entry.

3. Viral RNA, reverse transcriptase, and integrase enter the host cell.

4. Reverse transcriptase creates cDNA from viral RNA, which is transported into the nucleus.

5. Integrase integrates the cDNA into the host genome.

6. Viral RNA is transcribed and translated.

7. Two new RNA genomes and viral proteins coalesce at cell surface as a new virus particle forms by budding.

8. A mature HIV virion enters host circulation.

1. The gp160 spike on the HIV virion (gp120 + gp41) binds to CD4 on the host cell.

Figure 3. Artistic Conceptualization of the HIV Lifecycle Within a CD4+ Host Cell.
Following initial infection, an “acute phase” of HIV infection occurs, characterized by rapid proliferation of the virus and infection of many CD4+ cells (Figure 4). This initial viremia, peaking around 6 weeks post-exposure, results in high HIV titres in the plasma, spreading HIV throughout the body and into lymphoid organs with high populations of CD4+ cells. An initial telltale drop in the ratio of CD4+ to CD8+ T cells can be seen as many CD4+ cells are killed. Antibodies against HIV are produced during this time, but take a few weeks to reach diagnostic levels [31]. As many as 70% of infected individuals experience flu-like symptoms during this acute phase of HIV infection [32, 33].

During the initial viremia of the ‘acute phase’, integration of the viral genome into host DNA occurs, resulting in a widespread but clinically latent infection. This ‘clinically latent
phase’ typically lasts for many years, though it is well-established that viral replication and a gradual deterioration of the immune system are ongoing during this ‘latent phase’ [33]. At around six months, a ‘set-point’ occurs where CD4+ cell counts begin to decline gradually after rebounding from the dramatic decline seen during the ‘acute phase’. The plasma HIV RNA levels also stabilize around this time.

A ‘symptomatic phase’ heralds the end of the ‘latent phase’, the result of immune system collapse, as virtually all CD4+ T cells are lost. The AIDS-defining CD4+ T cell count is <200 cells per microlitre of blood, the threshold after which the appearance of common AIDS-defining illnesses begins, including the onset of non-Hodgkin’s lymphoma, Kaposi’s sarcoma, pneumocystis pneumonia (PCP), esophageal candidiasis, cryptococcal meningitis, and tuberculosis (TB), among others [34, 35].

1.1.3 Highly Active Antiretroviral Therapy (HAART)

HIV prevention and education efforts around the world have helped to reduce the number of new infections per year, but the increased accessibility of antiretroviral medications has saved an estimated 2.5 million lives in low- and middle-income countries, with 700 000 lives saved in 2010 alone [4]. In 2010, according to the United Nations AIDS (UNAIDS) initiative, 47% of eligible people worldwide were receiving antiretroviral medication, an increase of 1.35 million people over 2009 [4].

The first antiretroviral medication, azidothymidine (AZT, or zidovudine; ZDV), was first approved for use in patients with advanced HIV in 1987, some six years after the discovery of HIV [36]. Three more nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) followed
soon after: zalcitabine (ddC), didanosine (ddI), and stavudine (d4T) [36]. These dideoxy-NRTIs or “d-drugs” proved to be quite toxic, and are used only sparingly today [36].

HAART first became available in 1996 as an alternative to antiretroviral therapy mono- and dual drug regimens [37]. Triple-combination therapy and the “HAART era” began soon after the advent of different antiretroviral drug classes: non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Fusion inhibitors, entry inhibitors (also known as ‘entry/attachment inhibitors’), and integrase inhibitors were introduced more recently [36]. HAART is now considered ‘standard of care’, and is described as a combination of three or more drugs from two or more drug classes, with the goal of suppressing viral replication to ‘undetectable’ levels (generally under 50 copies/ml of blood). Current HAART therapy typically has less toxicity and side-effects than earlier iterations, it has become increasingly more manageable in terms of dosing frequency, and provides a better quality of life for patients [36]. Most current HAART combinations include two NRTIs and a third drug from one of the remaining drug classes; there are now single tablet, once-daily doses that combine three antiretrovirals, which have had greater compliance and therefore better efficacy [36].

HAART confers a median life expectancy to an HIV-positive patient as high as 41.5 years, a resounding increase when compared to the estimated life expectancy of 11 years in the case of the untreated HIV-positive individual [37-39]. Although great improvements in HAART tolerance have been made, there still remain several undesirable side-effects from antiretroviral therapy, such as headache and fatigue, diarrhea, lipodystrophy, hepatic steatosis, hepatotoxicity, lactic acidosis and hyperlactatemia, osteoporosis, hyperlipidemia, hypoglycemia and severe hypersensitivity reactions to some drugs (notably NNRTIs) [40, 41].
1.2 Hepatitis C Virus (HCV)

1.2.1 HCV Epidemiology

Hepatitis C was first characterized as “Non-A, Non-B Hepatitis” in the 1970s when patients receiving blood transfusions developed hepatitis that was ruled as not having been caused by Hepatitis A (*Picornaviridae, Hepatovirus*) or Hepatitis B (*Hepadnaviridae, Orthohepadnaviridae*) viruses. In 1989, the Hepatitis C virus (HCV) was identified from the *Flaviviridae* family of viruses, and was assigned to the genus *Hepacivirus*. HCV is estimated to infect approximately 170 million people worldwide [42]. The primary route of transmission throughout the developing world is unsafe medical practices, whereas HCV is primarily spread through intravenous drug use in the developed world [43].

Unlike HIV, HCV is highly infectious (up to ten times more infectious than HIV), and is stable outside of the host [44-46]. Accidental needle-stick injuries from abandoned syringes pose almost no risk of HIV transmission; HCV, in contrast, can remain infectious for weeks (temperature depending; 16 days at 25 °C, six weeks at 4 °C) on surfaces outside of the body [44, 47]. HCV can also be spread through sexual contact, vertical transmission (mother to child), body piercings and tattooing, and needle sharing; indeed, HCV and HIV share many commonalities in terms of routes of transmission.

According to the Public Health Agency of Canada, 242,500 Canadians are currently estimated to be infected with HCV. The Canadian Red Cross which previously provided blood services in Canada inadvertently caused more than 20,000 HCV infections (and 1,100 cases of HIV) via tainted blood products; an estimated 95% of Canadian haemophiliacs receiving blood transfusions contracted HCV between 1986 and 1990.
1.2.2 HCV Pathology

The natural course of HCV infection can take two different pathways: acute or chronic infection. Acute cases of Hepatitis C, though rare, are far preferable to having a chronic infection as acute infection almost never results in fulminant liver failure [48]. An estimated 15% of those who contract HCV spontaneously resolve their infections. These cases are more typically observed in young and female individuals [48]. Unfortunately, this leaves the vast majority of people exposed to HCV (the remaining 85%) with chronic Hepatitis C. Chronic Hepatitis C is diagnosed by persistent elevated aminotransferase (i.e. alanine transferase, ALT and aspartate aminotransferase, AST) and six months or more of detectable HCV RNA in the serum. Once this chronic state has set in, spontaneous resolution will not occur.

HCV infections are often asymptomatic; many people are unaware of their infection (as is in the case of HIV), and so it can be easily spread throughout a population. Figure 5 shows a representation of the natural course of HCV infection. The onset of serious complications or clinical symptoms from the viral infection varies. Morbidity may manifest as ‘scarring’ of the liver (fibrosis) and cirrhosis which tend to precede the development of hepatocellular carcinoma (HCC), though not everyone with HCV will develop HCC [49-51]. In fact, a patient infected with HCV can die of end-stage liver disease (ESLD) without ever having developed HCC. ESLD is a clinical term that refers to a state of advanced liver disease of various etiologies (for example, decompensated cirrhosis, alcoholic liver disease, chronic viral hepatitis, fatty liver disease) that has progressed to the point where liver transplantation is necessary to prevent death within a time frame of approximately six months or less. It is virtually synonymous with ‘liver failure’. There are no specific criteria for ESLD, though a model for end-stage liver disease, has
been formulated to predict mortality among potential liver transplant recipients [52]. In a recent study, the leading cause of mortality in a Canadian HIV and HCV co-infected cohort was ESLD [53].

![Figure 5. Natural Course of HCV Infection.](image)

Figure 5. Natural Course of HCV Infection. End Stage Liver Disease (ESLD) is the leading cause of death in patients with chronic HCV. Progression of chronic Hepatitis C is slow, and the development of cirrhosis typically takes between 20 and 30 years. Progression is dependent on several factors, including HIV co-infection, gender, age and alcohol intake.

Several factors can influence the progression of HCV to a chronic infection [51]. Co-infection with HIV is common among HCV positive individuals and represents a major risk factor for developing chronic HCV infection. A study by Thomas et al. in 2000 reported a reduced frequency of HCV clearance among individuals also infected with HIV compared to those infected with HCV alone [54]. Single nucleotide polymorphisms (SNPs) within the human genome can also influence HCV clearance; specifically, the SNP rs12979860 found upstream of
the IL28B gene [55]. Individuals containing a C/C isoform are shown to have a greater likelihood of HCV genotype 1 clearance compared to C/T and T/T isoforms. Of those individuals with the C/C isoform, HIV co-infection surprisingly has little impact on HCV resolution with both HIV positive and negative individuals successfully resolving their HCV infection (over 50% in both groups) [56].

There are 7 or more HCV genotypes that have been identified throughout the world (referred to as genotype 1-7), with various subtypes denoted by lower case letters (for example, HCV genotype 1b and HCV genotype 3a) [57]. A proposed phylogeny of the HCV genotypes and subtypes is shown in Figure 6. The World Health Organization (WHO) recognizes 11 distinct HCV genotypes globally [58]. Individuals infected with a particular HCV genotype can be infected with more than one subtype, and may be infected with a million or more ‘quasispecies’, mutated variants of the virus which helps to prevent the host from developing immunity to the virus [59, 60].

The most common genotype found in Canada is genotype 1 (~67%), followed by genotypes 3 (~22%), 2 (~9%) and 4 (~1%) [61, 62]. An association between HCV infection and steatosis of the liver has been established with different HCV genotypes which influences severity. HCV genotype 3 has been linked to the greatest degree of steatosis compared with other genotypes [63, 64].

Our understanding of the mechanisms underlying HCV replication is evolving. HCV is an enveloped, single stranded positive sense RNA virus that is approximately 9.6 kb long [65]. HCV has a reproduction life cycle of hours and is therefore highly replicative [66]. HCV can circulate in the body as a free virus and also in association with low density lipoprotein (LDL),
very low density lipoproteins (VLDL) and immunoglobulins [67]. Hepatocytes are the main cell type susceptible to HCV infection. Several cell surface receptors have been implicated as possible mediators of HCV entry into the cell including the LDL receptor [68], CD81 [69], scavenger receptor class B type I [70] and claudin-1 [71]. HCV gains access to the cytoplasm via endocytosis, followed by the uncoating of the HCV genome and release into the cytoplasm, the details of which are not well understood. Translation of the single stranded RNA then takes place in the cytoplasm of the cell using enzymes of the host cell. Translation of mRNA leads to the production of a polyprotein, which is subsequently cleaved into multiple proteins required for viral replication [reviewed in 72]. Mechanisms involved in the budding and release of the viral particle is not well understood however recent studies have suggested that the VLDL secretory pathway may be involved [73]. Because of the association of HCV with LDL/VLDL, the known role of the LDL receptor in internalization of HCV, and the potential involvement of lipid secretory pathways, factors involved in lipid metabolism are emerging as a potential therapeutic targets for combating HCV [74].
1.2.3 HCV Combination Therapy

Unlike HIV, HCV can be eliminated from the body. This “cure” is referred to as a sustained virologic response (SVR) which is characterized by undetectable HCV RNA levels.
from a patient six months (24 weeks) after completing HCV combination therapy [75]. Currently, treatment lasts for 48 weeks in patients with HCV genotype 1 (24 weeks for genotypes 2 and 3) [76-78], and consists of a combination of pegylated interferon (PEG-IFN), which enhances the body’s immune response to HCV, and the NRTI, ribavirin, intended to limit the replicative capacity of the virus. The effectiveness of HCV combination therapy varies according to HCV genotype. Genotype 1, the most commonly found HCV genotype in Canada, can be more difficult to treat compared to genotypes 2 and 3, reflecting the longer recommended treatment guidelines for genotype 1 (48 vs. 24 weeks). Unfortunately, treatment for HCV is not always successful and includes a number of undesirable side-effects. Severe anaemia causing haemolysis is the most common side effect, while flu-like symptoms as well as depression/anxiety are also common [79]. Overall, SVR rates for patients infected with HCV genotype 3 have been reported at 75-85% following a 48 week treatment regimen involving the combination of PEG-IFN and ribavirin, although the success rate is lower for those infected with genotype 1 (an average of 57% from 5 different studies) [78, 80].

There are currently two new drugs, boceprevir and telaprevir that have recently been added to the HCV treatment regime. Results from recently completed phase III clinical trials have demonstrated improved treatment outcomes and SVR when adding boceprevir and telaprevir to the existing regimen of PEG-IFN and ribavirin [81]. Both drugs are protease inhibitors and have been FDA approved for use in the USA as of May 2011. As mentioned, concerns about drug-induced hepatotoxicity have been raised when administering HCV combination therapy in conjunction with HAART. The addition of new drugs to the regime will therefore require additional studies to establish the impact of these drug combinations on liver toxicity in patients co-infected with HIV and HCV.
1.3 HIV/HCV Co-Infection

Transmission of HCV and HIV often share similar routes causing a number of affected individuals to be infected with both viruses. It is estimated that up to 30% of HIV infected individuals are also infected with HCV in the developed world. In Vancouver, British Columbia, the percentage of HIV positive individuals also infected with HCV is reported to be close to 50% [82, 83].

A leading cause of death in HIV/HCV co-infected individuals is ESLD resulting from chronic liver damage that has become irreversible and is a sign of imminent liver failure [84]. Liver diseases among those who are HIV positive are 11 times higher than the rest of the population [85]. Co-infection with HCV is thought to be a possible reason for this. In a 16-year study, the incidence of ESLD in HCV infected men was found to increase from 2.6% in HIV negative patients to 14.0% in those co-infected with HIV [86]. Another study found that in a population of HIV positive patients who died from ESLD, 93% were also co-infected with HCV [87]. Effective treatment of HIV using HAART has complicated the study of the effect of co-infection with HCV. In general, the use of HAART in co-infected individuals has benefited liver health [88-90] further highlighting the detrimental effect of co-infection with HIV, on HCV associated liver damage.

Liver biopsy continues to be the gold standard in assessing chronic liver damage. Elevated liver enzymes such as AST and ALT and other clinical markers typically included in a ‘liver haematology panel’ are used along with physical examination to determine if a liver biopsy is warranted [91]. Approximately 80 to 90% of the liver parenchyma must be destroyed before clinical symptoms of liver failure present [91]. A liver biopsy sample is used to determine the
extent of the pathology and a diagnosis of cirrhosis is typically confirmed using this method. Currently in British Columbia, it is required that physicians provide sufficient evidence of hepatic damage (“ALT level is more than 1.5 times the upper limit of normal on 2 consecutive occasions at least 3 months apart”) in order for the government to provide coverage of PEG-IFN and ribavirin [92]. The HCV genotype is also considered, as well as some contraindications (i.e. under the age of 18 years, active alcohol abuse, IDU etc.). Physicians involved in our study often included the pathology reports from the standard-of-care initial liver biopsy within their applications to provide evidence of chronic liver disease in the patients. The HIV/HCV co-infection was also highlighted, as some studies have shown a correlation between HIV co-infection and HCV disease progression [93]. However, other studies have failed to find a link between HIV infection and exacerbation of HCV disease progression or vice versa [45].

1.4 Treatment Associated Mitochondrial Toxicity

NRTIs are a major component of HAART, and are also present in HCV combination therapy in the form of ribavirin. In HIV, NRTIs are preferentially incorporated into an elongating DNA chain generated by HIV reverse transcriptase during viral replication. This prevents addition of subsequent nucleotides, terminating elongation of the DNA and interrupting viral replication. NRTIs have been implicated in mitochondrial toxicity [94]. Although multiple mechanisms behind NRTI-mediated mitochondrial toxicity have been suggested [95-97], a major cause of toxicity is the ability of NRTIs to inhibit the action of DNA polymerase γ, the key enzyme required for mitochondrial DNA replication in humans [98, 99]. Unwanted incorporation of NRTIs into the mitochondrial genome can lead to reduced replication and a net loss of
mtDNA [100]. Mitochondrial DNA polymerase γ has basic proofreading capabilities, but falls short of that of the nuclear DNA polymerases [101]. Increased mtDNA mutation has therefore also been suggested as a mechanism of mitochondrial toxicity [96, 102]. Other mechanisms of antiretroviral drug mitochondrial toxicity independent of the DNA polymerase γ have also been suggested [103].

Several studies over the last decade have addressed the hypothesis that NRTIs used in the treatment of HIV and HCV contribute to mitochondrial toxicity. Increased mitochondrial toxicity, as measured by a reduced blood mtDNA content, (mtDNA:nDNA ratio), was observed in HIV positive patients receiving antiretrovirals (including NRTIs) compared to both HIV positive patients not receiving antiretrovirals and healthy HIV negative controls [104]. Importantly, several of these HIV positive patients were receiving dideoxy-NRTIs which are particularly toxic, and are known to be a more effective inhibitor of DNA polymerase γ than other NRTIs [99, 105, 106]. However, new nucleotide analogues in development have minimal to no discernible mitochondrial toxicities in vitro [107]. Liver mtDNA depletion was also reported in patients receiving dideoxy-NRTIs compared to untreated HIV positive individuals [106]. More recently, our group saw no difference in the liver mtDNA content of HIV/HCV co-infected treated with HAART and those not on HAART [108]. In contrast to the previous studies, the majority of HAART-treated patients enrolled in this latter study were receiving non-dideoxy-NRTIs.

As mentioned, a large proportion of individuals infected with HIV are also infected with HCV [82]. Similar to HIV NRTIs, ribavirin has previously been suggested to negatively affect mtDNA synthesis [109]. The use of ribavirin as part of HCV combination therapy in addition to NRTIs already used in HAART prompted concerns of potentially increased mitochondrial
toxicity [110-112]. However, in a 2004 report by Ballesteros et al. examining peripheral blood cells from co-infected individuals receiving HAART (including dideoxy-NRTIs) found no evidence of mitochondrial toxicity following the addition of PEG-IFN and ribavirin for 24 weeks [113]. Blood mtDNA level may not reflect mtDNA content in other tissues.

Current guidelines for treating HCV in co-infected individuals however have changed PEG-IFN and ribavirin treatment from 24 to 48 weeks in an effort to increase rates of SVR. Furthermore, the effects of HCV combination therapy in addition to HAART on liver mitochondrial toxicity in co-infected patients has not been thoroughly investigated. Determining the impact of additional NRTIs used in current HCV combination therapy on liver toxicity will help inform current treatment guidelines for patients infected with HIV and HCV. With the recent introduction of the protease inhibitors telaprevir and boceprevir in the treatment of HCV, a complete understanding of the effects of PEG-IFN alpha and ribavirin on the co-infected and HAART-treated patient would be of great benefit moving forward [84, 114].
2. Objectives and Hypothesis

The treatment of both HIV and HCV simultaneously has led to concerns regarding the combined impact of these drugs on liver health. With increased survival and the rising number of HIV positive individuals also requiring management of their HCV infection as their liver disease progresses, understanding the impact of HCV combination therapy along with HAART is crucial for providing optimal treatment of co-infected patients.

In this study, we aimed to determine if the mtDNA content of liver hepatocytes from co-infected individuals changed in response to HCV combination therapy. We also sought to quantify changes in the ultrastructure of the hepatocytes, in particular if any changes to the mitochondria were evident.

The first of our three specific aims was to assess changes to the mtDNA:nDNA ratio following HCV combination therapy. The second was to investigate hepatocyte ultrastructure via Transmission Electron Microscopy (TEM) as an indication of the effects of HCV combination therapy. Our third aim was to compare available clinical data with our biochemical and cellular observations, through the blinded scoring of the liver biopsies by a Pathologist, and through haematological data and physician’s findings recorded in patient charts.

My central hypothesis was that *HCV combination therapy may result in hepatocellular changes consistent with either increased damage or a healing response in these HIV and HCV co-infected patients*. The results of this study will contribute to our understanding of the impact of HCV combination therapy in HIV-infected individuals, including those on HAART.
3. Materials and Methods

3.1 Study Population

This “HIV/HCV Co-infection” study was an open, prospective observational cohort study funded by a CIHR grant (CIHR (HOP-75347), title: Mitochondrial toxicity in HIV/HCV co-infection antiviral therapy, PI Hélène Côté). The procedures and protocols used in this study were approved by the University of British Columbia and Providence Health Care Research Ethics Board (UBC-PHC REB # H03-50055).

Inclusion Criteria:

To be enrolled in this study, participants were to be:

- Positive for antibodies against both HIV and HCV
- HCV therapy naïve
- HAART naïve, or have been stably on-HAART or off-HAART for 6 months prior to start of study
- 19 years of age or older at time of enrolment
- be able and willing to give informed consent

Exclusion Criteria:

Prospective participants to this study were excluded at the time of enrolment if they were:

- Infected with Hepatitis B, or have metabolic liver disease
- Currently suffer from an opportunistic infection (AIDS)
- Pregnant or breastfeeding, or of childbearing age and not willing to avoid becoming pregnant during the study period
- Currently receiving the HIV drug didanosine (ddl)

Patients for whom their treating physician had already planned a liver biopsy as part of their standard care were invited to participate in this study. Study participants were enrolled after
providing written consent following a study orientation at St Paul’s Hospital. Study participants consented to undergo a double-liver biopsy rather than the standard-of-care single-liver biopsy. This liver biopsy is done in order to assess fitness for the HCV combination therapy via a Pathologist’s assessment of the gross liver specimen. In addition, participants who initiated HCV therapy following their initial biopsy agreed to undergo another double-liver biopsy upon completion of the HCV-combination therapy, this time for research purposes only.

3.2 Liver Biopsies

Two ultrasound-guided, 10-gauge/18 mm needle core biopsy tissue samples were taken in the morning (between 10 and 11AM) from a single incision at both the pre- and post-HCV therapy biopsy appointments by a radiologist at St Paul’s Hospital. The subjects were instructed to fast overnight prior to the procedure, and provided us information about their last food intake prior to the biopsy. For the pre-treatment biopsies, the first sample was given to hospital pathology and the second to research while for post-treatment biopsies, the first sample was given to research and the second to hospital pathology.

The biopsy tissue destined for hospital pathology was placed in standard 10% formalin phosphate-buffered solution to undergo further processing for paraffin embedding and sectioning, following standard histopathology protocols. Slides were stained with haematoxylin and eosin (H&E), Masson’s Trichrome and Periodic Acid Schiff (PAS) stains, and were made available to our study through the kind cooperation of Ms. Sue Finley, Technical Leader of Anatomic Pathology at St Paul’s Hospital.
The biopsy taken for research purposes was transported immediately, dry, in a sterile specimen container to the Centre for Excellence in HIV and AIDS (St Paul’s Hospital) for processing. Within five minutes of the biopsy collection, the 18 mm-long tissue sample was sliced with a sterile, disposable scalpel into 4 separate pieces of roughly equal length, approximately 4.5 mm. Two of these pieces were immediately frozen (dry) in a sterile micro-centrifuge tube placed in a -80 °C freezer for future nucleic acid or protein studies. The third aliquot was immersed in “RNAlater” RNA Stabilization Reagent (QIAGEN) and kept overnight at 4 °C. In the morning, the “RNAlater” solution was removed, and the specimen frozen and stored dry in a -80 °C freezer. The fourth quarter was processed for standard Transmission Electron Microscopy (TEM) (see section 3.7).

3.3 Pathologist’s Scoring of Liver Biopsy Slides

Light microscopy slides were coded and de-identified using a simple numerical system so that a certified Pathologist, Dr David Schaeffer, could score and grade the study participant’s slides in a blinded fashion for hepatic pathology. Using the modified Ishak-Knodell Histological Activity Index (HAI) staging and grading system (Table 1), the Pathologist scored each coded slide set and also gave commentary and an estimated burden of macrovesicular steatosis as a percentage (in 5 to 10% increments). This particular scoring system was preferred for its specificity as well as simple, numerical total score; by addition of the individual scores for Periportal Interface Hepatitis, Confluent Necrosis, Focal Lytic Necrosis, Portal Inflammation and Stage, a total score of 24 is possible. This overall score allows for quick comparison of liver pathology between biopsies in pathology reports, and the breakdown to the individual
necroinflammatory scores and the stage provide specific information in several distinct aspects of possible liver pathologies.

Table 1. Ishak-Knodell-Modified HAI Scoring System.

<table>
<thead>
<tr>
<th>Necroinflammatory Scores:</th>
<th>Score</th>
<th>Focal Lytic Necrosis, Apoptosis, &amp; Focal Score</th>
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<tbody>
<tr>
<td></td>
<td>Periportal Necrosis</td>
<td>Interface Hepatitis</td>
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<td>Confluent Necrosis</td>
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Modified Staging:

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<tr>
<th>Stage</th>
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<tr>
<td>No fibrosis 0</td>
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<tr>
<td>Fibrous expansion of some portal areas, with or without short fibrous septa 1</td>
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<tr>
<td>Fibrous expansion of most portal areas, with or without short fibrous septa 2</td>
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<tr>
<td>Fibrous expansion of most portal areas with occasional portal to portal bridging 3</td>
</tr>
<tr>
<td>Fibrous expansion of portal areas with marked bridging (both portal to portal, and portal to central) 4</td>
</tr>
<tr>
<td>Marked bridging (both portal to portal, and portal to central) with occasional nodules (incomplete cirrhosis) 5</td>
</tr>
<tr>
<td>Cirrhosis, probable or definite 6</td>
</tr>
</tbody>
</table>
3.4 Nucleic Acid Extraction

Total DNA and RNA were extracted simultaneously from frozen biopsy tissue using the QIAGEN AllPrep DNA/RNA Extraction Mini Kit (QIAGEN; Mississauga, ON), according to the kit’s handbook protocol “Simultaneous Purification of Genomic DNA and Total RNA from Animal Tissues”. A QIAGEN “TissueRuptor” was used to homogenize the tissue, with plastic disposable probes that were treated with bleach, “RNaseZap” (Invitrogen) and diethylpyrocarbonate (dep-C) treated water between each re-use. Some biopsies were collected by previous students and in some instances, total DNA was extracted from frozen tissue using the QIAmp DNA Mini Kit while the biopsy specimen stabilized with “RNAlater” was preferentially used for DNA and RNA extraction, in accordance with the recommended protocol except that AE Buffer (10mM Tris, 0.5mM EDTA, pH 9.0) was used to elute the DNA rather than Buffer EB (10mM Tris, pH 8.5).

3.5 Mitochondrial DNA Content Assay

Total DNA extracted from the liver tissue was amplified by real-time quantitative polymerase chain reaction (qPCR) to separately quantify a mitochondrial DNA-encoded gene (Cytochrome C Oxidase subunit I (CCOI)) and a single copy nuclear DNA-encoded gene (Accessory Subunit of Polymerase Gamma (ASPG) as previously described [104, 115]. Each separate qPCR reaction was run in duplicate in a LightCycler 480 (Roche; Laval, QC), and the resultant amplified DNA were quantified against a standard curve made from serial dilutions (15.5 to 1,550,000) of a plasmid containing the region amplified. The second-derivative
maximum value generated for each amplification reaction was used and replicates were accepted if within the standard curve and within 20% of each other. The 10 µl qPCR reactions were run using the LightCycler 480 Probes Master Kit (Roche) and contained 2 µl of the sample DNA, 5mM of magnesium (Mg$^{2+}$), 1 µM of each primer (ASPG or CCOI), 0.2 µM of the fluorescein-labeled hybridization probe, and 0.4 µM of the Red 640-labelled hybridization probe. An initial hot-start for 10 min at 95°C was followed by 45 PCR cycles (1 - denaturation (5 min at 95°C), 2 - annealing (10 s at 60°C), 3 - extension (5 s at 72°C)). For the final cycle, the last extension step lasted for 7 min. Signal acquisition of the FRET fluorescence was performed post-annealing. Two negative controls (extraction buffer and elution buffer) and two internal controls were included in each qPCR run. The results of this assay were expressed as a ratio of the mtDNA (mean value of the two replicates) to nuclear DNA (mean value of the two replicates).

3.6 Mitochondrial DNA Deletion Assay

Using a “Long PCR” assay established in our laboratory adapted from Stringer et al. [116], mtDNA deletion events resulting in mtDNA genomes of less than 16.6 kb were visualized in an agarose gel. Briefly, the mtDNA copy number of each DNA extract was quantified using the CCOI real-time quantitative PCR (qPCR) assay described in section 3.5. The entire mtDNA genome was amplified in two fragments with the primers: MT16535F (5’-GCCACACGTTCCCCTAAATAAGA-3’) + MT8388R (5’-CGGTTAGTATTGTCATTTCAC-3’), and MT7988F (5’-CTCCTTGACGTGACAATCGAGT-3’) + MT708R (5’-GGGATGCTTGCATGTGTAATCTTTAC-3’) with the Expand Long Range dNTP pack kit (Roche). Each long template PCR reaction contained 200,000 copies of mtDNA.
as template. The amplification was carried out in a MyCycler (Bio-Rad, Hercules, CA, USA) under the following reaction conditions: 93°C/2min followed by 35 cycles of 93°C/10s, 58°C/30s, and 68°C/6min. Beginning at cycle 11, an additional 20s extension time was added to each cycle. The PCR products were run on a 0.75% agarose gel stained with ethidium bromide and their banding pattern analysed with UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). For each fragment, the pixel density of full-length product and deleted bands was quantified and the deletion burden [(deleted band/total bands pixel density)*100] was calculated. The mtDNA score was defined as the sum of the two fragments’ deletion burden and ranged from 0 to 200.

3.7 Processing of Biopsy Tissue for Transmission Electron Microscopy (TEM)

The quarter aliquot of the liver biopsy taken for research purposes was sliced by scalpel in a droplet of 2.5% glutaraldehyde, into 5 to 6 pieces of approximately 0.75 to 1mm³ each. This small size was necessary in order to achieve optimal fixative penetration of the tissue. These small pieces were then transferred into a tube of freshly made 2.5% glutaraldehyde in a 0.1M sodium cacodylate buffered solution at pH 7.4 for two hours at 4 °C.

Following fixation and washing with 0.1M sodium cacodylate buffer for at least an hour, the specimens were post-fixed in a 0.1M sodium cacodylate buffered solution of 1% (v/v) osmium tetroxide and 1% (v/v) potassium ferrocyanide for 1.5 hours at room temperature, with mild agitation (plastic-capped 2 dram glass EM vials were placed on an angled rotating platform at approximately 1 rpm to facilitate fixative penetration).
The osmium tetroxide solution, and subsequent washes (distilled water), were disposed of in accordance with current UBC (Risk Management Services) guidelines. Following 3 washes to remove unbound osmium tetroxide, the pieces of tissue were dehydrated through a gradient of increasing concentrations of acetone in water (30%, 50%, 70%, 90%), and were then infiltrated with increasing concentrations of epoxy resin or “Epon” (Eponate 12 Kit with BDMA, PELCO®, Ted Pella Inc.; Redding, CA, USA) in acetone up to a 2:1 ratio of Epon to acetone. This mixture was left to evaporate overnight at room temperature in a fume hood, gradually increasing the relative concentration of Epon while decreasing the concentration of acetone by evaporation. The following morning, the specimens were transferred into fresh 100% Epon, and placed in a 45 °C oven for 24 hours. The next morning the oven temperature was increased to 60 °C for an additional 24 hours to complete polymerization of the resin.

Once the embedding procedure was complete, a single piece of tissue was randomly selected. Each block was trimmed, and then sectioned with a diamond knife on a LEICA EM UC6 ultramicrotome into “thick” reference sections (0.5 μm) for light microscopy and then “thin” (60 nm) sections for TEM. Thin sections were picked up on Formvar-coated mesh grids and stained with 2% uranyl acetate (6 to 10 minutes at room temperature) followed by Reynold’s lead citrate (4 to 7 minutes at room temperature) [117].

The reference sections for light microscopy were stained with Toluidine Blue-O. In some cases the reference sections confirmed that a particular biopsy had missed the liver and therefore no hepatocytes were present. In one instance, the biopsy contained only muscle and capsular tissues, missing the hepatocytes altogether.
3.8 TEM

Transmission electron microscopic viewing and image capture was done with a FEI Tecnai 12 TEM at 80 keV. The sites to be sampled on the tissue section were determined in a standardized random fashion. At these sites, images were captured at 2400x, 5800x, 18500x and 46000x magnification. To select a cell profile, the first complete hepatocyte cell profile observed in the upper left-hand corner of the grid square was used. An image of this cell profile was captured at 2400x in order to estimate cell volume. The cytoplasm of the same cell was then imaged at 5800x for point counting (see section 3.11). It was often necessary to “stitch” several images in order include the entire cell at this magnification. The 5800x images were “stitched” together using the Adobe Photoshop CS (version 8.0) automated photo-merge feature to create a single image for morphometric analysis. Next, the magnification was increased to 18500x without moving the specimen grid from its original position, in order to capture an area of the hepatocytes’ cytoplasm with its mitochondrial profiles. If this image did not contain mitochondria, the magnification was decreased to 5800x times, and the grid was moved by one field of view, and the process was repeated. Any complete mitochondrial profiles in the field of view were then imaged at 46000x in order to do a line intercept estimation of surface to volume ratios of cristae to matrix (see sections 3.12).

3.9 Stereology

Several stereological morphometric techniques were utilized to estimate structural characteristics of hepatocytes in a quantitative fashion. To estimate the volume of hepatocytes
from 2-dimensional images, the Star Volume technique was employed [118]. Point counting was used to estimate the relative volume fractions of selected organelles (mitochondria, lysosomes, nucleus) and cytoplasmic inclusions (such as lipids and glycogen). To estimate the surface to volume ratios of cristae to matrix from mitochondrial profiles, the line-intercept and point counting methods (as outlined in sections 3.11 and 3.12) were employed.

3.10 Cell Size by Star Volume

To determine whether volumes of hepatocytes were changing, the mean hepatocyte volume was estimated for each sample prior to and following treatment. The star volume stereological technique allows estimation of the three-dimensional volume of a cell or structure from a two-dimensional image. As defined by Gundersen et al., the star volume is “the mean volume of all parts of an object which can be seen unobscured in all directions from a particular point” [119]. The particular point in the grid was selected in a standardized random fashion. To estimate the volume of an irregularly shaped cell, the mean of 16 radii measured from an interior selected point within the cell to the plasma membrane was applied to the following mathematical formula: where \( r \) = the mean value of 16 measured radii

\[
Star\ Volume = \frac{4}{3}(\pi r^3)
\]

where \( r = \text{the mean value of 16 measured radii} \)

From a particular point, a 16 radial line grid was overlaid on a 2400x image of a complete cell profile, and each radius was measured using Image-Pro Plus (version 4.5.0.29, Media
Cybernetics Inc., Bethesda, MD, USA) (Figure 7). The “point” which serves as the origin of the 16 radii measured was selected in a standardized random fashion. A 90 pixel by 90 pixel grid of potential points was placed over the image, and each point falling in the cell was assigned a number as if in an array. A random number between 1 and the number of points that fit within the confines of the cell profile was generated using online software (Random.org, Randomness and Integrity Services Ltd., Dublin, Ireland) and the corresponding point was then used as the origin for the 16 radii measured. The tissue had been randomly oriented by the embedding process.

As described previously by our group [108], the calculated number of cell profiles required to produce a mean star volume with a stable coefficient of variation for each biopsy was 21. Therefore, 21 cell profiles at 2400x were measured for each sample.
Figure 7. Example of Dot Matrix and Star Grid Overlays over a 2400x image of a Hepatocyte for Estimation of the Star Volume. A 90 x 90 pixel dot matrix (red) is overlaid. A dot falling on the hepatocyte is selected, through random number generation, as the origin for the subsequently overlaid 16 radii star grid (yellow). Radii are then measured using the ImagePro software and averaged, allowing an estimate of hepatocyte volume.
3.11 Organelle Populations by Point Counting

The volume fractions of organelles and other cytoplasmic inclusions within the hepatocyte were estimated using point counting. A 40 pixel x 40 pixel grid was superimposed on a complete cell profile at 5800x as in Figure 8. It was then recorded whether each point ‘hit’ one of the following structures within the cell:

- Nucleus (green)
- Glycogen (white)
- Lipid (magenta)
- Mitochondria (blue)
- Lysosome (not pictured)
- Cytoplasm, including vesicles, endoplasmic reticulum, peroxisomes and Golgi (yellow)

A seventh category of “other” or “undefined” was also included, but was not used. The number of hits of these categories divided by the total number of points hitting the image of the cell constituted an estimate of the volume fraction for each of the organelles or other inclusions within the hepatocytes. However, as each section represents only a small portion of the entire hepatocyte, the mean of the volume fractions derived from many sections through multiple cells were calculated. As previously determined [108], the sample size required to obtain a stable coefficient of error was 6 cell profiles. Therefore, 6 complete cell profiles at 5800x magnification were counted for each sample. The numbers of mitochondrial profiles and lysosomal profiles occurring in each section were also recorded.

Although peroxisomes only make up an estimated 1% or less of the volume fraction of hepatocytes [120] they were often difficult to distinguish with certainty. As a result, the volume fraction of peroxisomes was included in the category of “cytoplasm, including vesicles,
endoplasmic reticulum and Golgi”. Similarly, the endoplasmic reticulum (both smooth and rough) and elements of the Golgi apparatus were not separately quantified. It was not possible to distinguish endoplasmic reticulum from other endomembranes at this magnification. For this reason the volume fractions of both the endoplasmic reticulum and elements of the Golgi apparatus could be somewhat under- or over-estimated. Therefore, we did not attempt to directly quantify these organelles.

Occasionally, a bit of debris on the section obscured a region of the cell under a point; in these rare instances, the point was scored as cytoplasm. Therefore, the point was included because it represented a volume within the cell, and exclusion could potentially skew the estimate of total volume in which the organelles or inclusions occurred.
Figure 8. A Portion of a Hepatocyte Profile at 5800x Point Counted for Organelles. The different coloured markers describe what each point on the overlaid matrix “hit”. The ImagePro software used tallies the hits for each category. A volume fraction of the organelles in a hepatocyte can then be estimated. (Green = nucleus; Blue = mitochondria; White = glycogen; Magenta = lipid; Yellow = cytoplasm)
3.12 Line Intercept

To assess the ultrastructure of mitochondria for changes with treatment, the cristae surface-to-volume ratio was estimated using the line-intercept technique [121]. As with point counting, all points falling within the mitochondrial profile were recorded, in order to assess potential changes to the ultrastructure of the mitochondria. The following structures were point counted:

- Mitochondrial matrix
- Inter membrane spaces of cristae
- Inner mitochondrial membrane and cristae membrane
- Outer mitochondrial membrane
- Inclusion bodies (also known as “Mitochondrial Matrix Granules”)

The grid overlay used for both assessment of the mitochondrial volume fractions by point counting and for the line intercept technique consisted of “L”-shaped lines, which were laid over mitochondrial images at 46 000x. The intersections of the two lines forming the “L”-shaped lines served as points for volume estimates; the horizontal component of the “L”-shaped lines served as lines for surface estimates (Figure 9). Similarly, at this same magnification, the line intercepts were counted; an intercept was counted each time the line crossed cristae membrane. Interceptions of a portion of a horizontal line with cristae membranes or the inner mitochondrial membrane are counted as estimates of surface area. In many cases, a line would transect a crista profile, resulting in two hits. Because cristae may be “cisternal” in shape they may be seen “en face”. If a line crossed a crista en face, only one hit was counted.
Surface density of the cristae was then calculated using the following formula:

\[ Surface\ Density = \frac{(2 \sum i)}{(x \sum P)} \]

where:

- \( i \) = the number of interceptions
- \( x \) = the length of the linear grid line (0.121 µm)
- \( P \) = the number of points hitting the mitochondria

As previously described [108], the sample size for mitochondria required to generate a stable coefficient of error was 40 profiles. Therefore, for each sample 40 mitochondrial profiles were analysed. The rationale for choosing the line-intercept method over a cycloid grid was based on the absence of anisotropy in mitochondria or cristae. The property of being directionally dependent must be taken into consideration for some specimens. A systematic bias could be introduced by using a linear grid or an anisotropic specimen. However, as the orientation of cristae within the mitochondrion is isotropic, use of a linear grid was appropriate [122]. This was previously confirmed by Matsukura et al. for the measurement of cristae surface density [108].
Figure 9. Two Mitochondrial Profiles at 46000x with Overlaid Grid showing a Point Counted Mitochondrial Matrix (lower left mitochondrion) and Line Intercepts with Cristae (upper right mitochondrion). Perimeters were measured by tracing the outer membrane of the mitochondrial profiles using ImagePro.
3.13 Mitochondrial Profile Perimeters

The perimeters of the 40 mitochondrial profiles used to calculate cristae surface-to-volume ratios were also measured using a manual tracing measurement feature of the Image-Pro Plus software. Along with the volume fraction of mitochondria and the cristae surface-to-volume ratio, the mean perimeter of the mitochondrial profile in each sample provides a mechanism for detecting any changes in the sizes of mitochondrial profiles before and after treatment. Taken together with the cristae surface-to-volume ratio and the volume fraction, the perimeters of the mitochondrial profile can help to examine the potential for mitochondrial hypertrophy.

3.14 Qualitative Analysis

In addition to the quantitative stereological measurements, qualitative descriptive data were collected at the TEM and the light microscope levels. At the TEM level, the distributions of glycogen and lipid within the cell were noted, as was the presence and contents of tertiary lysosomes. At the 2400x and 5800x magnifications, neighbouring cells and cell-cell junctions were examined; bile cannaliculi, Kupffer cells, and sinusoids and their contents were described when remarkable. Collagen fibres were also noted when they occurred in the extracellular matrix. Putative autophagosomes and nuclear abnormalities were also noted in some circumstances. Membranous whorls in the mitochondria, a visually striking feature, were also imaged and described in detail.
3.15 Statistics

Mean values were used for all morphometric measurements. Statistical analyses were selected in consultation with a mathematical modeler (Dr. Oliver Günther, PROOF Centre of Excellence at St Paul’s Hospital). The Wilcoxon signed ranked test was used to compare the two groups before and after treatment. Statistical calculations were computed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
4. Results

4.1 Study Population

Of the 80 study participants enrolled, 63 underwent double-biopsies for assessment, and 17 were deceased or lost to follow-up prior to their first biopsy. Of the 63 participants who were assessed and underwent a double biopsy, 20 were not recommended for HCV therapy, and 23 delayed treatment or were lost to follow-up. Of the remaining 43, 26 initiated HCV therapy. Of those, 7 patients were either still on HCV therapy or had not yet started, and 6 had completed therapy but had not yet returned following treatment for the post-HCV therapy biopsies at the time of data analysis and were considered lost to follow-up. Two (2) patients declined post-HCV therapy biopsies. Finally, 11 study participants underwent both pre- and post-HCV therapy biopsies. These details are summarized in Figure 10.

Of these 11, 2 were not on HAART within 6 months of enrolment in the study, including one who was HAART-naïve, and remained off-HAART throughout the study period. Eight (8) were on HAART at the time of enrolment, and 1 initiated HAART during HCV combination therapy for a total of 9 who were considered to be on-HAART. The present study includes these 11 individuals. Their characteristics are presented in Table 2. The 11 study subjects that returned for a post-HCV therapy biopsy had routine blood-work assessed at the BC-CfE (BC Centre for Excellence in HIV/AIDS at St. Paul’s Hospital). Post-hoc analysis from available chart data provided such parameters as days of HAART exposure, the CD4+ T-lymphocyte counts, HIV viral load, liver function and transaminase measurements, and whether or not the subject had experienced an AIDS-defining event.
Figure 10. Summary of Study Population. Of all patients consented, pre and post biopsies were collected from a total of 11 patients (10 male and 1 female). Of these 11 biopsies, 9 were from patients on HAART while 2 were from patients not on HAART.
Table 2. Demographics of Study Participants who Underwent HCV Combination Therapy and Both Pre- and Post-HCV Combination Therapy Liver Biopsies.

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Sex</th>
<th>Age at pre-HCV therapy biopsy</th>
<th>Reported HCV genotype</th>
<th>HAART status</th>
<th>Identified risk factors for HIV and HCV</th>
<th>HCV combination therapy outcome</th>
<th>Reported serious adverse effects during HCV combination therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>COINF 03</td>
<td>male</td>
<td>33</td>
<td>3a</td>
<td>Off</td>
<td>IDU</td>
<td>SVR</td>
<td>none</td>
</tr>
<tr>
<td>COINF 16</td>
<td>male</td>
<td>42</td>
<td>1a</td>
<td>On</td>
<td>unknown</td>
<td>SVR</td>
<td>none</td>
</tr>
<tr>
<td>COINF 24</td>
<td>female</td>
<td>36</td>
<td>3</td>
<td>On</td>
<td>unknown</td>
<td>SVR</td>
<td>none</td>
</tr>
<tr>
<td>COINF 25</td>
<td>male</td>
<td>43</td>
<td>1a</td>
<td>On</td>
<td>unknown</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>COINF 36</td>
<td>male</td>
<td>59</td>
<td>3a</td>
<td>Naive</td>
<td>IDU</td>
<td>Failed</td>
<td>none</td>
</tr>
<tr>
<td>COINF 44</td>
<td>male</td>
<td>43</td>
<td>1a</td>
<td>On</td>
<td>IDU</td>
<td>Failed</td>
<td>none</td>
</tr>
<tr>
<td>COINF 47</td>
<td>male</td>
<td>53</td>
<td>1a</td>
<td>On</td>
<td>IDU</td>
<td>Failed</td>
<td>none</td>
</tr>
<tr>
<td>COINF 49</td>
<td>male</td>
<td>47</td>
<td>1</td>
<td>On</td>
<td>IDU</td>
<td>#</td>
<td>none</td>
</tr>
<tr>
<td>COINF 51</td>
<td>male</td>
<td>61</td>
<td>1b</td>
<td>On</td>
<td>MSM</td>
<td>Failed</td>
<td>anaemia (blood transfusions req’d)</td>
</tr>
<tr>
<td>COINF 52</td>
<td>male</td>
<td>42</td>
<td>1a</td>
<td>On</td>
<td>MSM</td>
<td>SVR</td>
<td>none</td>
</tr>
<tr>
<td>COINF 61</td>
<td>male</td>
<td>33</td>
<td>1b</td>
<td>On</td>
<td>MSM</td>
<td>SVR</td>
<td>none</td>
</tr>
</tbody>
</table>

SVR = sustained virologic response (HCV negative (by PCR) after 6 months or more post-treatment)

* = patient interrupted therapy on own accord, citing self-diagnosed depression. Did not achieve SVR.

# = patient was HCV negative at 4 months but did not have follow up blood work done to confirm SVR.

IDU = intravenous drug use

MSM = men who have sex with men
4.2 Assessment of Liver Pathology Before and After HCV Combination Therapy

The IK-HAI pathology scoring was done by a single, blinded certified pathologist, Dr David Schaeffer, and was used to determine the difference in liver pathology before and after HCV combination therapy. Pathology scores revealed a significant reduction in liver pathology following treatment (Table 3, Figure 11). Of the 11 subjects from whom pre and post biopsies were obtained, 4 could not be properly scored due to unavailable histological slides or insufficient tissue to score. One additional subject, COINF 51, received additional drugs known to be detrimental to liver pathology and was excluded from these analyses (discussed below). All of the remaining six (6) subjects included in the pathology scoring demonstrated a significant ($P = 0.03$) reduction in liver pathology (Figure 11). In summary, these data suggest that HCV combination therapy may be beneficial to liver health in subjects co-infected with HIV and HCV. As mentioned previously (see section 3.3), the IK-HAI pathology scores can be further broken down into sub-scores (portal inflammation, fibrosis, stage etc.). Although it did not reach significance ($P = 0.089$), the “stage” score also suggested a trend of overall improvement in liver health (Table 3, Figure 12).

In addition to the IK-HAI pathology scores, the pathologist also estimated the overall steatotic burden of the biopsies, including both macro- and micro-vesicular steatosis. Although not significant ($P = 0.058$), a trend was observed suggesting a possible decrease in liver steatosis following HCV treatment (Table 4, Figure 13).

COINF 51 experienced a marked increase in estimated steatosis (from 0% to 40%). This subject’s medical chart indicated that he had been treated for neurosyphilis and for PCP prophylaxis during the HCV combination therapy period with the drugs Penicillin-V
(phenoxyethylpenicillin) and Septra. The latter is contraindicated in patients with liver problems [123] and has been known to cause fatal complications including hepatitis, cholestatic jaundice and hepatic necrosis, elevation of serum transaminase, alkaline phosphatase and bilirubin [123, 124]. Further, the serum biochemistry performed on this patient showed a dramatic increase in both AST and ALT following treatment (alkaline phosphatase was not available following HCV therapy in this subject). Because of the additional drugs which may have adversely impacted his liver, this subject was excluded from our analyses.
### Table 3. Pathologist's Scoring of Liver Biopsies Before and After HCV Therapy

<table>
<thead>
<tr>
<th>STUDY I.D.</th>
<th>Pre-HCV Therapy Pathology Scores</th>
<th>Post-HCV Therapy Pathology Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IK-HAI total score (/24)</td>
<td>Portal Inflammation (/4)</td>
</tr>
<tr>
<td>COINF 03</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>COINF 16</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>COINF 24</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>COINF 25</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>COINF 36</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>COINF 44</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 47</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 49</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>COINF 51</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>COINF 52</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 61</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2</td>
</tr>
</tbody>
</table>

n/a indicates a missing hospital pathology slide that could not be recalled from storage, the refusal of the subject for a second needle core taken at time of post-HCV therapy biopsy, or insufficient biopsy tissue for the Pathologist to score.
Figure 11. Change in IK-HAI Pathology Scores Following HCV Therapy. Liver biopsies from subjects receiving HCV therapy demonstrated a significant decrease in IK-HAI pathology score following treatment. (*$P = 0.0355$, Wilcoxon signed rank test). Overlapping data points are slightly offset for visualization purposes.

Figure 12. Change in the Modified Staging Score Following HCV Therapy. Although not significant ($P < 0.089$), the modified staging score showed a trend towards a decrease following HCV combination therapy. (ns = not significant, Wilcoxon signed rank test). Overlapping data points are slightly offset for visualization purposes.
Table 4. Pathologist Estimated Percentage of Steatosis Before and After HCV Therapy

<table>
<thead>
<tr>
<th>STUDY I.D.</th>
<th>Pre-HCV Therapy Estimated % Steatosis</th>
<th>Post-HCV Therapy Estimated % Steatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>COINF 03</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>COINF 16</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>COINF 24</td>
<td>10</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COINF 36</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>COINF 44</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 47</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>COINF 49</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>COINF 51</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>COINF 52</td>
<td>n/a</td>
<td>5</td>
</tr>
<tr>
<td>COINF 61</td>
<td>40</td>
<td>0</td>
</tr>
</tbody>
</table>

*n/a* indicates a missing hospital pathology slide that could not be recalled from storage, the refusal of the subject for a second needle core taken at time of post-HCV therapy biopsy, or insufficient biopsy tissue for the Pathologist to score.

Figure 13. Change in the Pathologist Estimated Percent Steatosis Following HCV Therapy. Although not significant (*P* = 0.058), a trend towards an overall decrease in estimated steatosis was observed following HCV combination therapy. (*ns* = not significant, Wilcoxon signed rank test). Overlapping data points are slightly offset for visualization purposes.
4.2.1 HAART Parameters and HIV-related Clinical Laboratory Assays

Table 5 below shows the HIV-related parameters for these patients. Interestingly, regardless of HAART usage, the HIV viral load appeared to decrease (or remain undetectable) during HCV therapy for all participants. Overall, there was no significant change in CD4+ T-lymphocyte counts for each subject at the time of their pre- and post-HCV therapy liver biopsies.

Table 5. HAART Exposure, CD4+ Counts and HIV Viral Loads Before and After HCV Therapy

<table>
<thead>
<tr>
<th>STUDY I.D.</th>
<th>HAART status at Pre-HCV therapy Biopsy</th>
<th>Exposure to HAART at time of Pre-HCV Therapy Biopsy (days)</th>
<th>CD4 Count at Pre-HCV Therapy Biopsy (cells/mm³)</th>
<th>CD4 Count at Post-HCV Therapy Biopsy (cells/mm³)</th>
<th>&lt;200 CD4 cells/mm³ Nadir</th>
<th>HIV Viral Load at Pre-HCV Therapy Biopsy (copies/mL)</th>
<th>HIV Viral Load at Post-HCV Therapy Biopsy (copies/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COINF 03</td>
<td>OFF</td>
<td>1895</td>
<td>340</td>
<td>160</td>
<td>n/a</td>
<td>≥100010</td>
<td>72100</td>
</tr>
<tr>
<td>COINF 16</td>
<td>NAIVE*</td>
<td>0</td>
<td>400</td>
<td>290</td>
<td>**</td>
<td>47900</td>
<td>101</td>
</tr>
<tr>
<td>COINF 24</td>
<td>ON</td>
<td>2563</td>
<td>210</td>
<td>290</td>
<td>n/a</td>
<td>undet.</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 25</td>
<td>ON#</td>
<td>399</td>
<td>350</td>
<td>470</td>
<td>YES</td>
<td>undet.</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 36</td>
<td>NAIVE</td>
<td>0</td>
<td>400</td>
<td>290</td>
<td>YES</td>
<td>≥100010</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 44</td>
<td>ON</td>
<td>1030</td>
<td>390</td>
<td>240</td>
<td>n/a</td>
<td>undet.</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 47</td>
<td>ON</td>
<td>1064</td>
<td>480</td>
<td>650</td>
<td>YES</td>
<td>undet.</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 49</td>
<td>ON</td>
<td>408</td>
<td>380</td>
<td>550</td>
<td>YES</td>
<td>76</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 51</td>
<td>ON</td>
<td>2900</td>
<td>210</td>
<td>290</td>
<td>YES</td>
<td>undet.</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 52</td>
<td>ON</td>
<td>155</td>
<td>470</td>
<td>770</td>
<td>YES</td>
<td>114</td>
<td>undet.</td>
</tr>
<tr>
<td>COINF 61</td>
<td>ON</td>
<td>288</td>
<td>430</td>
<td>530</td>
<td>NO</td>
<td>undet.</td>
<td>undet.</td>
</tr>
</tbody>
</table>

*HAART-naive at Pre-HCV therapy biopsy; initiated HAART during HCV therapy

# ON-HAART at Pre-HCV therapy biopsy; hiatus from HAART during HCV therapy

** Yes, but physician indicates that reading is likely erroneous due to medications taken at the time

"undetectable" HIV viral load defined as a count of less than 50 copies/mL by qPCR

"undet." = undetectable
4.2.2 Liver-related Clinical Laboratory Assays and Ratios

Liver function and transaminase blood-work panels were performed routinely as part of standard care. Table 6 shows clinical laboratory blood-work tests for the study participants before and after HCV therapy. Parameters measured varied highly between study participants, as did the frequency of tests prior to, during and following HCV therapy. Data was sparse in some cases, due largely to the variations in panels ordered. Nevertheless, a comparison of several liver-related parameters could still be performed.

As seen in Table 6 and Figure 14A, two subjects had marked increases in their aspartate transaminase (AST) levels following HCV therapy (COINF 51 and 61), suggesting that liver injury may have occurred during HCV therapy. Neither of these two patients began HCV therapy with clinically elevated AST (defined as >47 IU/L [125]). The alanine transaminase (ALT) level of COINF 61 at the post-HCV therapy biopsy was more than 10 times the pre-HCV therapy measurement. Overall, there was an even split between those who increased and those who decreased in transaminase levels following HCV therapy.

Alkaline Phosphatase was also measured in these subjects (Table 6, Figure 7). Only two (2) of the seven (7) patients compared had clinically elevated levels; one of which normalized following HCV therapy, and no post-therapy test was available for the other.
Table 6. Before and After HCV Therapy Transaminases and Alkaline Phosphatase Test Results from Dates Closest to the Pre- and Post-HCV Therapy Liver Biopsies

<table>
<thead>
<tr>
<th>STUDY I.D.</th>
<th>AST (IU/L; normal &lt;35 IU/L)</th>
<th>ALT (IU/L; normal &lt;49 IU/L)</th>
<th>AST/ALT Ratio</th>
<th>Alkaline Phosphatase (IU/L; normal 35-120 IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>COINF 03</td>
<td>30</td>
<td>35</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>COINF 16</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 24</td>
<td>46</td>
<td>n/a</td>
<td>60</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 25</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>COINF 36</td>
<td>55</td>
<td>52</td>
<td>63</td>
<td>75</td>
</tr>
<tr>
<td>COINF 44</td>
<td>50</td>
<td>22</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>COINF 47</td>
<td>44</td>
<td>62</td>
<td>46</td>
<td>93</td>
</tr>
<tr>
<td>COINF 49</td>
<td>51</td>
<td>37</td>
<td>70</td>
<td>37</td>
</tr>
<tr>
<td>COINF 51</td>
<td>31</td>
<td>122</td>
<td>36</td>
<td>277</td>
</tr>
<tr>
<td>COINF 52</td>
<td>26</td>
<td>26</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>COINF 61</td>
<td>32</td>
<td>128</td>
<td>23</td>
<td>237</td>
</tr>
</tbody>
</table>

Underlined ratios indicate a 'significant predictor of severe liver fibrosis' (Angulo et al. 2003)
Bold font indicates clinically relevant elevated value
Reference ranges are those of BC Biomedical Laboratories (108)

Figure 14. Transaminases Before (o) and After (●) HCV Combination Therapy. No significant difference was observed in AST or ALT levels following HCV combination therapy. (ns = not significant, Wilcoxon signed rank test).
Figure 15. Alkaline Phosphatase Levels Before (○) and After (●) HCV Combination Therapy. No significant difference was observed in alkaline phosphatase levels following HCV combination therapy. (ns = not significant, Wilcoxon signed rank test).
4.3 Mitochondria

4.3.1 Mitochondrial to Nuclear DNA Ratios and Mitochondrial DNA Deletions

Nucleoside analogues, such as the NRTIs commonly employed in HAART, and ribavirin, a component of HCV combination therapy, have been shown to be toxic to the mitochondria and to reduce the mtDNA content (mtDNA:nDNA ratio) in peripheral blood leukocytes, likely through inhibition of DNA polymerase gamma [104]. We extracted total DNA from frozen liver tissue and measured the mtDNA:nDNA ratios before and after HCV therapy.

There was no significant difference in the mtDNA:nDNA ratios of the 7 participants before and after HCV therapy (Figure 16, Table 7). One subject, COINF 03, had a marked increase in his mtDNA:nDNA ratio following treatment. This subject was HAART-experienced but was off-HAART at the time of his pre-HCV therapy biopsy, and remained so during HCV therapy. It is notable that of the subjects studied, this patient had a high HIV viral load both at the pre- and post-HCV therapy biopsy (≥100010 (above upper limit) at pre-, and 72100 at post-HCV therapy), and had a sub-200 CD4+ cell count near the time of his post-HCV therapy biopsy.

Also in respect to possible DNA polymerase gamma inhibition, potential mitochondrial DNA deletions were also investigated using long PCR (see section 3.6). Unfortunately, this method used a relatively large quantity of the extracted total DNA from the liver biopsies, and was not applied to all samples. In the few samples studied, including some samples from subject biopsies who did not qualify for HCV therapy, we did not see evidence of truncated mtDNA
genomes. To preserve remaining DNA, we abandoned this part of the investigation in favour of future studies.

Figure 16. Mitochondrial to Nuclear DNA Ratio Before (○) and After (●) HCV Combination Therapy. No significant difference was observed in the mtDNA to nDNA ratio in liver biopsies from patients before and after HIV/HCV combination therapy (ns = not significant, Wilcoxon signed rank test).

Table 7. mtDNA:nDNA Ratios Before and After HCV Combination Therapy

<table>
<thead>
<tr>
<th>Study ID</th>
<th>mtDNA:nDNA Ratios</th>
<th>Commentary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>COINF 03</td>
<td>395</td>
<td>1200</td>
</tr>
<tr>
<td>COINF 16</td>
<td>428</td>
<td>304</td>
</tr>
<tr>
<td>COINF 24</td>
<td>644</td>
<td>322</td>
</tr>
<tr>
<td>COINF 36</td>
<td>419</td>
<td>442</td>
</tr>
<tr>
<td>COINF 44</td>
<td>386</td>
<td>575</td>
</tr>
<tr>
<td>COINF 47</td>
<td>476</td>
<td>364</td>
</tr>
<tr>
<td>COINF 49</td>
<td>628</td>
<td>615</td>
</tr>
<tr>
<td>COINF 52</td>
<td>291</td>
<td>589</td>
</tr>
<tr>
<td>COINF 61</td>
<td>290</td>
<td>406</td>
</tr>
</tbody>
</table>
4.3.2 Ultrastructural Analyses - Mitochondria

Liver biopsies were analysed morphometrically by TEM to identify any changes in volume fractions or surface areas of ultrastructural constituents of hepatocytes from subjects before and after HCV combination therapy. Qualitative observations were also made regarding the organelles of the hepatocytes and their ultrastructure.

The average volume fraction of mitochondria per cell did not change following HCV combination therapy (Figure 17A). While the majority (6 of 8) of subjects showed a reduction in the mitochondrial volume fraction following treatment, two (2) subjects demonstrated an increase. The greatest increase in mitochondrial volume fraction occurred in subject COINF 52. COINF 52 was the only subject treated with Lopinavir as part of his HAART regimen; whether this drug is partly responsible for this marked increase in mitochondrial volume fraction is unknown. This subject also experienced the largest increase in CD4+ T-lymphocyte count at the time of the post-HCV therapy biopsy, reaching levels considered to be within the normal range for a healthy, HIV-negative adult [126]. Future studies including a greater number of subjects treated with Lopinavir may be worthwhile.

Since mitochondria can have different morphologies at different stages in the cell cycle, including an ‘indeterminate’ mixture of both the “fragmented” and “tubular” morphologies for the majority of the G1 phase (including G0 phase), the latter part of S phase, and the whole of G2 phase [127], we also counted the number of discrete mitochondrial profiles seen in each hepatocyte that was point counted for volume fractions. Overall, the average number of mitochondrial profiles per cell did not vary significantly ($P = 0.11$, Figure 17B).
We examined the density of inner mitochondrial membranes by estimating the surface-to-volume ratio or cristae to matrix by the line intercept method. The majority (6/7) of subjects investigated saw an increase in the cristae surface-to-volume ratio following combination therapy, with the exception of COINF 52 who decreased dramatically (Figure 17C). When COINF 52 was excluded, the cristae surface-to-volume ratio of the remaining subjects reached significance (p = 0.0125). A possible explanation for this finding is seen in studies of human cell lines showing that the inner mitochondrial membrane mass increases during G₁ and S phase of the cell cycle [128], whereas the mitochondrial volume fraction and outer membrane increases proportionally with the cytoplasm at a consistent rate [129].

Point counting of the mitochondrial contents enabled us to detect potential swelling of the cristae and inter-membrane space by volume fraction alone or in conjunction with cristae measurements. Again, no significant difference was observed in these measurements (Figure 17D).

Mitochondrial perimeters were measured along with the morphometric assessment of the mitochondrial contents at 46 000x (Figure 17E). Mitochondrial matrix inclusions, also known as mitochondrial matrix granules, were counted as well. Although much debate still exists as to the purpose and the exact composition of these commonly seen inclusions [130], a considerable number of inclusions were noted in one case (blinded) during TEM image capture at 46,000x, prompting us to count them (Figure 17F). This outlier was later determined to be COINF 36, the only HAART-naïve subject in this study. COINF 36 showed a 4.7 fold decrease in the number of mitochondrial matrix granules following combination therapy. As noted earlier, COINF 52 was the only subject to have shown an increase in matrix inclusions following treatment, nearly
doubling (from an average of 0.28 granules per mitochondrial profile to 0.50). With the exclusion of COINF 52, these findings approach statistical significance ($P = 0.016$).

**Figure 17. Ultrastructural Analyses of Mitochondria.** All parameters were measured from TEM images of liver biopsies before (○) and after (●) HCV combination therapy. (A) Mitochondrial volume fractions, (B) Number of Mitochondrial Profiles per Cell Profile, (C) Mitochondrial Cristae Surface-to-Volume Ratio, (D) Volume Fraction of the Inter-Membrane Space of the Mitochondria, (E) Mitochondrial Perimeter measurements and (F) Number of Matrix Inclusions per Mitochondrial Profile. (ns = not significant, Wilcoxon signed rank test)
4.4 Hepatocyte Volumes Before and After HCV Combination Therapy

Hepatocyte volumes were estimated before and after HCV combination therapy. Although a decrease in the average volume of hepatocytes was observed following treatment, it did not reach significance (Figure 18).

Figure 18. Hepatocyte Volumes. Hepatocyte volumes were estimated by star volume before (○) and after (●) HCV combination therapy. Volumes did not change significantly \( (P = 0.461) \) following HCV combination therapy, by achievement of sustained virologic response or by HCV genotype. (ns = not significant, Wilcoxon signed rank test)
4.5 Glycogen

Glycogen, the storage form of glucose, is found in many cells in the body. The liver alone is unique in its ability to mobilize glucose from glycogen to other parts of the body when required. The glycogen load in the ‘normal’ human liver has been estimated to be approximately 6% of its wet weight [131].

Subjects in this study were required to fast overnight prior to their biopsy scheduled for the following morning. Where possible, confirmation of fasting and information on the pre-fasting meal was garnered. In addition to glycogen levels fluctuating with food intake, circadian rhythms [132] and gender can also play a role in glycogen storage in the liver. Therefore, biopsies were scheduled for the same time of day. The lone female in our group had the most glycogen of all the subjects before combination therapy, and the second least amongst the subjects following treatment. The outlier with the lowest estimated glycogen following treatment was COINF 25, whose glycogen estimates decreased by over 31%. This subject is known to be an occasional user of illicit drugs (crack cocaine and crystal methamphetamine).

Thus, glycogen quantities were estimated before and after HCV combination therapy, as possible indicators of hepatocyte metabolism. These data demonstrated that HCV combination therapy did not result in any significant difference in the average volume fractions of hepatocyte glycogen stores in liver biopsies taken before and after treatment (Figure 19). These results do not suggest that there was any a dramatic change in hepatocyte metabolism of glycogen following HCV combination therapy.
Figure 19. Glycogen Volume Fractions Before (o) and After (●) HCV Combination Therapy. Glycogen volume fractions show no significant difference following HCV combination therapy. (ns = not significant, Wilcoxon signed rank test)
4.6 Lipids

The liver plays a central role in the body’s lipid metabolism. Aberrant lipid accumulation resulting in steatosis occurs in many liver diseases, and hepatic steatosis in which lipid content of the liver exceeds 5-10% of its mass, can occur as a result of a variety of diseases and circumstances (alcohol abuse, toxic chemicals, obesity, diabetes, pregnancy, etc.). Chronic HCV infection can be associated with abnormal lipid deposition and steatosis in hepatocytes [133], and the acronym VASH (viral-associated steatohepatitis) has been coined to highlight the similarities in these two diseases [134].

HCV has several distinct genotypes, two of which (genotypes 1 and 3) are common in Vancouver, and are included in our study population. HCV genotype 3 has been associated with increased lipid burden compared to other genotypes [63, 64]. A previous study in our laboratory comparing liver biopsies taken from HIV/HCV co-infected subjects on and off HAART [108] confirmed a significant increase in the amount of intracellular lipid in the hepatocytes of subjects infected with HCV genotype 3.

In our study, the volume fraction of hepatocyte lipid decreased significantly ($P = 0.0078$) in all subjects following HCV combination therapy (Figure 20). This decrease was independent of HAART-experience, gender, age, success of HCV therapy (achievement of SVR), and also of HCV genotype. Strikingly, subject COINF 36, the only subject who was HAART-naïve, decreased only slightly (3.9 to 3.7%) following treatment yet the statistically significant $p$ value by Wilcoxon signed rank test remained significant ($P = 0.015$) even if this subject was excluded. These results strongly suggested that HCV combination therapy contributed to a decrease in
stored lipid in the hepatocytes of patients co-infected with HIV and HCV regardless of achievement of sustained virologic response to HCV or of HCV genotype.

Figure 20. Lipid Stores in Co-Infected Hepatocytes Before and After HCV Combination Therapy.
(A) Pre-HCV combination therapy hepatocyte profile with substantial lipid accumulation and post-HCV combination therapy hepatocyte from the same subject (COINF 44). Images taken at 5800x. Scale bars = 2 μm. (B) Volume fractions of lipid in liver biopsies from patients before (○) and after (●) HCV combination therapy. (*P < 0.05; P = 0.016, Wilcoxon signed rank test)
4.7 Lysosomes

The lysosomes of a cell function as a disposal system for damaged or unwanted organelles and end-stage metabolites (via autophagy), phagocytosed foreign bodies such as viruses or bacteria, and macromolecules.

Lysosomes in long-lived cells such as neurons are often prominent; ultrastructurally secondary and tertiary lysosomes are often loaded with pigmented osmiophilic materials and thus stain near black when osmium post-fixed. We estimated the volume fraction of secondary and tertiary lysosomes in the hepatocytes before and after HCV combination therapy.

No significant difference was observed in the volume fraction of lysosomes following HCV combination therapy (Figure 21A). The overall volume fraction of lysosomes were relatively small in both the pre-treatment and post-treatment samples, ranging from 1.3 to 7.4% and the majority of the subjects showed an increase in lysosomal volume fraction (6/8).

We also counted the number of individual lysosomes in each point-counted cell profile (Figure 21B). Interestingly, the only subject to decrease in number of lysosomes following treatment was again COINF 52. With the exclusion of COINF 52, the increase in the number of lysosomes following combination therapy was significant ($P = 0.016$ from 0.055).
Figure 21. Ultrastructural Analyses of Lysosomes. Neither (A) volume fractions nor (B) the number of lysosomes per cell profile were significantly different following HCV combination therapy (before (o) and after (●) HCV combination therapy). However, when COINF 52 is excluded, the number of lysosomes counted per cell profile increased significantly ($P = 0.016$ from 0.055, Wilcoxon signed rank test).
4.8 Nuclei and Cytoplasm

The nuclear volume fraction of hepatocytes can vary, as an estimated 30-40% of adult human hepatocytes are polyploid (binucleate, tetraploid, octoploid) [135]. It was necessary to estimate nuclear volume fractions separately in these cells so that organelle volume fractions would be of the cytoplasm. Likewise, the volume fraction of ‘cytoplasm’, which included cytosol and other organelles (endoplasmic reticulum, Golgi, etc.) not directly quantified here, was estimated.

HCV combination therapy resulted in no significant change in the nuclear volume fractions of cells (Figure 22A). The majority of individuals demonstrated an overall decrease in nuclear volume fraction following treatment but this trend did not reach significance. In addition, no significant difference was observed in the average volume fraction of cytoplasm following HCV combination therapy (Figure 22B).

Figure 22. Nuclear and Cytoplasm Volume Fractions. Volume fractions of nucleus (A) and cytoplasm (B) did not change significantly (ns = not significant, Wilcoxon signed rank test).
4.9 Binucleate Cells

The normal human liver is estimated to have between 30 and 40% polyploidization of its hepatocytes. To ascertain if the degree of binucleation changed following HCV combination therapy, we estimated the number of binucleated hepatocytes by estimating the number of binucleate cells per square micron on scanned light microscopy slides. There was no change in the degree of binucleation following HCV therapy, though this method does not take into account 4N or 8N polyploid nuclei (Figure 23). It was of interest to examine the number of binucleate cells as it has been shown that HCV infection can disrupt the mitotic spindle checkpoint function of the cell cycle [136].
4.10 Qualitative Observations

Not all observations were quantitative, and in several instances remarkable ultrastructure was observed and described here. Structural changes in the nucleus and mitochondria, and the qualities of other organelles and cellular components (such as lysosomes, lipid and glycogen), were imaged and described.

4.10.1 Senescence in Hepatocytes – Presence of Vacuolated Nuclei

Although a rare occurrence in normal liver, nuclear vacuolation in hepatocytes has been observed in non-alcohol-related fatty liver diseases (including chronic Hepatitis B and C infections) using light microscopy and is believed to be a marker of senescence [137]. Aravinthan et al. associated the presence of vacuolated hepatocyte nuclei with cell cycle arrest and nuclear DNA damage [137]. Transmission electron micrographs of nuclei with invaginations containing lipid and glycogen (Figure 24) were observed in subjects COINF 03 and COINF 49, both prior to HCV combination therapy.

In these cells, huge invaginations of the nuclear envelope are dominated by large lipid droplets and some attendant cytoplasm. A lysosome is clearly visible along with the lipid in the bottom cell of Figure 24. This would appear to be a nuclear ‘vacuole’ at the light microscopic level given that the lipids would be extracted by solvents and inadequate resolution of light microscopy.
Figure 24. **Vacuolated Nuclei as Markers of Cellular Senescence.** Lipid-containing nuclear invaginations from subjects COINF 03 (TOP) and COINF 49 (BOTTOM) prior to HCV combination therapy by TEM.
4.10.2 Autophagy – Putative Autophagosomes

Putative autophagosomes meeting the criteria outlined by Eskelinen et al. [138] were occasionally observed in the co-infected hepatocytes. In Figure 25 (TOP), ribosomes can be clearly seen in the inner edge of the rough endoplasmic reticulum surrounding the mitochondria, satisfying the conditions put forth for autophagy by Eskelinen et al. In the lower image of Figure 25 (BOTTOM), the encapsulated mitochondrion shows a disorganized outer membrane structure and cristae, though present, are irregular in shape, volume and localization within the organelle. It is most likely that this autophagosome has already fused with a primary lysosome as the inner ER membrane is also irregular or absent. The process of digestion of the mitochondrion appears to have begun. A ‘normal’ mitochondrion immediately next to the enveloped one stands in sharp contrast in its membrane structure.

Autophagy is considered a normal process in the liver, essential for cellular homeostasis [139]. Autophagy can also be utilized in a cell during starvation to destroy redundant organelles for efficiency. However, glycogen present within the field of view in both cells, even though the subject would have been fasting prior to the biopsy, suggest that starvation is not a factor.
Figure 25. Autophagy and Putative Autophagosomes. Possible evidence of autophagy in two individuals prior to HCV therapy. Scale bar = 0.2 μm.
4.10.3 Mitochondrial Matrix Granules and Membranous Whorls

Mitochondrial matrix granules, once thought to be cation sinks (Ca$^{2+}$, in particular), have been shown by Barnard and others to be composed of phospholipid regardless of the tissue of origin, and to associate with the mitochondrial inner membrane [140]. Current theories suggest that granules represent stores of phospholipid for formation of cristae, and that they are confluent with the inner mitochondrial membrane. Although not indicative of any known pathology, Somlyo et al. noted that more mitochondrial granules were seen in damaged vascular tissues and ischemic myocardium [141]. An example of these granules is shown in Figure 26.

Barnard and Ruusa included several TEM images of mitochondrial matrix whorls, including one which described the “membrane-like internal arrangement” of a “large matrix granule” [140]. This “unfurling” of a once densely-coiled matrix granule was similar to many of the membrane whorls seen in this study (Figure 27 and Figure 28), though their shape, size and localization varied.
Figure 26. Mitochondrial Matrix Granules. A hepatocyte mitochondria showing two electron-dense matrix granules (arrowheads). Image taken from a pre-HCV combination therapy subject’s biopsy (COINF 34). Scale bar = 0.2 μm.
Figure 27. Membranous Whorls of Various Forms in Mitochondria. Examples of membranous whorls found in hepatocyte mitochondria from multiple patient biopsies. A) an inner mitochondrial membrane whorl. B) and C) whorls at the junction of two mitochondrial profiles. D) large whorl of the outer mitochondrial membranes.
Figure 28. Large Mitochondrial Membranous Whorl. A large membranous whorl in a mitochondrial matrix nearly devoid of 'normal' cristae.
4.10.4 Tertiary Lysosomes

Tertiary lysosomes are end stage lysosomes. Prominent tertiary lysosomes with a variety of contents ranging from densely osmiophilic to membranous were noted in many of the subjects. The lysosomes were in various stages of maturation; in a few cases, large and osmiophilic spherical lysosomes remained in the cytoplasm of hepatocytes with few surrounding organelles and an absence of glycogen. These tertiary lysosomes were evident in both pre and post-HCV combination therapy biopsy samples. Figure 29A depicts a few tertiary lysosomes containing swirls of osmiophilic membranes in subject COINF 44 prior to HCV combination therapy. Figure 29B is an example of the apparent separation of tertiary lysosomes and glycogen. In several instances densely packed glycogen was spatially separate from clusters of tertiary lysosomes in close proximity to one another, suggesting polarity of the hepatocytes from basal surfaces to bile canaliculi [142, 143]. Figure 29C and D are both from subject COINF 52 following HCV combination therapy, showing perhaps early tertiary lysosomes which are more spherical in shape, yet with varying contents. Endoplasmic reticulum, both smooth and rough, and glycogen granules are present.
Figure 29. Tertiary Lysosomes. Examples of tertiary lysosomes found in hepatocytes from multiple patients both before and after HCV combination therapy. A) Examples of cluster-like tertiary lysosomes from a pre-HCV combination therapy subject. B) Spatial segregation of glycogen (upper left) and a field of tertiary lysosomes (lower right). C) Tertiary lysosomes adjacent to a mitochondrial profile at right in a post-HCV combination therapy subject. D) Tertiary lysosomes with varied contents in a post-HCV combination therapy subject.
4.10.5 Lipid Droplets – Macrovesicular and Microvesicular Steatosis

Large lipid droplets apparently deforming nearby nuclei were seen in several hepatocytes. Figure 30A is from a pre-treatment biopsy (subject COINF 03), and Figure 30B is from a post-treatment biopsy (subject COINF 24). Figure 30C shows a mitochondrion with abnormal architecture with a large lipid droplet possibly originating from the mitochondrion.
Figure 30. Lipid Droplets. Examples of hepatocyte lipid droplets in biopsies from multiple patients both before and after HCV combination therapy. A) and B) large lipid droplets deforming adjacent nuclei. C) a large lipid droplet interacting with the outer membrane of an atypical mitochondrion; cristae membranes at the centre of the mitochondrial profile forming loose membrane whorl.
5. Discussion

5.1 Major Findings

5.1.1 Lipid Decreases in Co-Infected Hepatocytes Following HCV Combination Therapy

The group of all subjects studied experienced a statistically significant decrease in the lipid volume fraction of biopsied hepatocytes following HCV combination therapy. This was regardless of HCV genotype, HAART-exposure, differing HAART regimes, HIV viral load, CD4 count, success of HCV combination therapy, age or gender.

To our knowledge, this is the first time that a significant decrease in the lipid content of HIV/HCV co-infected hepatocytes following HCV combination therapy has been shown. The TEM-based morphometric analyses employed provided a rigorous, quantitative estimate of the intracellular volume fraction of lipid in hepatocytes. Although lipid droplets seen in these hepatocytes varied in size, they were often less than 5 micrometers in diameter, suggesting that the majority of steatosis was microvesicular in nature. As was shown previously [144], the incidence of macrovesicular steatosis (as defined by the displacement or distortion of the nucleus by large lipid droplets) was occasionally noted but was by no means the norm in our study. In fact, the majority of the lipid content in the hepatocytes would have been difficult to accurately quantify via light microscopy with its limit of resolution. The vast majority of the lipid droplets seen were electron translucent in appearance, owing to the light osmium staining acquired during processing. A few lipid droplets were more electron dense, though not quite as darkly stained as lipid droplets of neighbouring hepatic stellate cells known to contain vitamin A [145, 146]. Nevertheless, the great majority of the lipid observed appeared to be very lightly stained,
suggesting a predominance of saturated fatty acids of neutral lipids as the double bonds of unsaturated fatty acids react with osmium tetroxide.

Lipid metabolism in HIV/HCV co-infected patients is of particular interest because of recent reports of the ability of HCV to “highjack” lipid trafficking and metabolism, leading to increased synthesis, decreased secretion and catabolism of lipids in the liver [reviewed in 73]. HCV has been suggested to circulate in the blood attached to lipoproteins [147, 148]. Entry of HCV into cells is believed to be accomplished through endocytosis via receptors responsible for cholesterol absorption such as NPC1L1, prompting the use of NPC1L1 inhibitors as a possible therapeutic for combating HCV infection [149]. Our findings demonstrating a significant decrease in hepatocyte lipid volume fraction following HCV combination therapy would therefore be consistent with a decrease in this effect of HCV infection.

Some groups have suggested that HAART influences lipid accumulation in co-infected individuals, and have even speculated that a decrease in the capacity of the mitochondria to carry out β-oxidation of fatty acids could be to blame [150, 151]. However, this has been refuted by other groups [152-154]. In a previous study from our laboratory, we showed that there was no difference in the lipid volume fraction of hepatocytes taken from subjects taking HAART versus those who did not.
5.1.2 Pathologist’s Scores

Paraffin-embedded, formalin-fixed sections of liver biopsies were also examined to assess the degree of liver pathology present before and after HCV combination therapy. Liver pathology was assessed using the IK-HAI pathology scoring system [155]. The Pathologist’s assessment revealed a significant improvement in liver health following treatment as indicated by the IK-HAI scores. This was consistent with an overall beneficial effect of HCV combination therapy to liver health. Pathologist Dr David Schaeffer’s blinded scoring indicated that again, regardless of HCV genotype, HAART, HIV viral load, CD4 count, achievement of an SVR, age or gender, the course of HCV combination therapy seemed to benefit the liver. Indeed, even a truncated treatment period less than the standard-of-care 48 weeks of HCV combination therapy (as recommended for HIV-HCV co-infected individuals) resulted in an improvement in the assessment of liver pathology. Subject COINF 25 discontinued HCV combination therapy at his own volition at 24 weeks. The subject’s medical chart indicates that he experienced depression, and he related verbally that he ‘quit partway’ because subcutaneous injection of the medication was distressingly reminiscent of his prior injection drug habit. Although subject COINF 25 failed to achieve a SVR, his post-treatment liver biopsy showed an improvement in liver health. Therefore, despite concerns of increased hepatotoxicity, the administration of treatment for both HIV and HCV was beneficial to the subjects in our study.

When the amount of steatosis was semi-quantitatively estimated by the Pathologist, an overall reduction was also observed following HCV combination therapy. This reduction approached but did not reach statistical significance ($P=0.058$). This could be explained by the
inability to accurately resolve the full extent of microsteatosis via light microscopy as used by the Pathologist to score the biopsies.

5.1.3 mtDNA:nDNA Ratio

Both HAART and HCV combination therapy typically involve the use of multiple NRTIs. Ribavirin is the sole NRTI used in HCV combination therapy. Previous studies investigating mtDNA:nDNA ratios in peripheral blood cells suggested that the use of NRTIs in HAART is associated with mitochondrial depletion and toxicity in these cells [156]. The use of the dideoxy-NRTIs or “D-drugs” was suggested to be particularly toxic in this regard. D-drugs are less commonly used now in HAART as a result, and in Canada are used almost exclusively as an option for patients struggling with HIV viral resistance [157, 158]. Studies showing mtDNA depletion in the liver, lymphocytes and kidney as a result of HAART involved the use of D-drugs [106, 159].

A previous study from our lab examining HIV and HCV co-infected hepatocytes from subjects on and off-HAART suggested that there was no difference in the ratio of mtDNA:nDNA. Similarly, we did not observe a difference in the mtDNA:nDNA ratio of hepatocytes before or after HCV combination therapy.
5.1.4 Morphometric Analyses

When examining the ultrastructure of hepatocytes following HCV combination therapy, we found no significant changes to mitochondrial ultrastructure. These results are consistent with our corresponding observations that HCV combination therapy failed to cause a significant increase in mitochondrial toxicity in the form of mtDNA depletion. A previous study has reported an association with mtDNA depletion and changes in mitochondrial ultrastructure [160]. The lack of evidence of ultrastructural changes in our study combined with our corresponding mtDNA:nDNA data and improved pathology scores further suggest that HCV combination therapy was actually beneficial to the liver, and failed to induce increased mitochondrial toxicity even when used concurrently with HAART.

The number of mitochondrial matrix granules per mitochondria appeared to decrease following HCV combination therapy ($P = 0.090$). As this decrease did not reach significance, a larger sample size might take this trend to significance. However interesting, the exact significance of these inclusions is currently unknown. In 1966, Ashworth et al. [161] first showed that lipid was a component of mitochondrial matrix granules, and Barnard et al. [140] thought that the granules were precursors to mitochondrial inner membranes. More recently, Jacob et al. [162] examined the matrix granules of heart muscle mitochondria and determined that they indeed contain phospholipids and cytochrome c oxidase, among other constituents, and they showed that in stimulating conditions, the granules move toward and are incorporated into the mitochondrial inner membranes. They concluded that mitochondrial matrix granules in the heart form the structural basis of “a regulatory mechanism”, a reservoir of lipids with “which cells can cope with a high and sudden energy demand” [162]. It should be noted however that we
did not observe a change in the cristae surface to volume ratio in our study, nor any correlation between the matrix granules and our cristae density data. Further investigations into the effects of HCV combination therapy on these inclusions may be worth considering in future studies examining hepatocyte ultrastructure in co-infected individuals. A corresponding decrease in matrix inclusions would be in accord with an improvement in hepatocyte health, a corollary to the improvement in liver health as evidenced by decreasing pathology scores, and possibly provide new insights into the meaning of these inclusions.

The volume fractions of glycogen, lysosomes, nuclei and cytoplasm did not change following HCV combination therapy. When glycogen was assessed, a rather sharp decrease was seen in COINF 24, the lone female in this study. As intracellular glycogen is known to fluctuate with menstrual cycle [132], it is possible that this discrepancy is unrelated to the present study. Lysosomes play an important role in the cell by collecting and managing metabolic waste and unwanted organelles. A change in the amount of lysosomes in hepatocytes may be reflective of a change in the cell’s metabolism. In our study however, hepatocyte lysosome volume fractions also failed to change significantly following HCV combination therapy. Our observations failed to provide evidence that these cellular activities were altered following HCV combination therapy. Similarly, no significant differences were observed in the volume fractions of the cytoplasm or nucleus following HCV combination therapy. These results, along with our observations that hepatocyte cell volume and the number of binucleate cells did not change significantly following HCV combination therapy suggests that changes in cell size could not have been responsible for our observed significant decrease in lipid volume fraction.

In summary, despite the limited sample size, we feel that these results are consistent with the hypothesis that HCV combination therapy results in an improvement of liver health
consistent with a repair response. We found ultrastructural evidence of reduced steatosis as well as an improvement in overall liver health as determined by IK-HAI pathology scores, consistent with HCV clearance. Importantly, we also failed to find evidence of increased hepatocyte and mitochondrial toxicity following HCV combination therapy, suggesting that the therapy used here is effective and had an overall beneficial effect on the livers of these subjects.

5.2 Study Limitations

5.2.1 Sample Size and Liver Heterogeneity

The effects of HCV combination therapy on ultrastructural characteristics of hepatocytes was undertaken in this study despite the relatively small number of subjects. Although a significant reduction in hepatocyte lipid content was observed, a greater number of study participants would strengthen the results and might have led to the identification of additional significant ultrastructural changes following treatment. For example, p-values of less than 0.1 (though above 0.05), were observed in the number of mitochondrial inclusions ($P = 0.090$), number of lysosomes per cell profile ($P = 0.078$) and cytoplasm volume fraction ($P = 0.078$) in hepatocytes following HCV combination therapy. Pathology scores identified trends in the IK-HAI staging ($P = 0.089$) and percent steatosis ($P = 0.058$) of liver biopsies following treatment. Both results failed to reach significance, possibly due to a limited number of subjects, or in the case of lipids (steatosis) may have been due to limitations in the study design, namely the ability to resolve the lipid at the light microscopy level and the semi-quantitative system used to estimate overall steatosis.
Although liver biopsies provide a valuable method for assessing the status of the liver before and after treatment, they represent only a small fraction of overall volume of the liver. Liver damage resulting from HCV infection and/or treatment does not necessarily occur uniformly throughout the liver [163]. An unequal distribution of liver pathology could complicate the interpretation of the results. In spite of this limitation, Pathologists have and continue to use this as an important method for assessing liver health. For the assessment of liver fibrosis, liver stiffness as measured by FibroScan® technology (http://www.fibroscan.co.uk/) holds promise as a possible alternative to traditional liver biopsy, though some limitations have also been recognized for this technique [164].

5.2.2 Study Population and Patient Enrollment

Examination of hepatocyte ultrastructure before and after treatment, while useful in determining the effect of treatment, does not allow for a comparison between diseased and healthy (never infected) hepatocytes. Determining whether or not changes to hepatocyte ultrastructure are beneficial or detrimental is difficult to discern without healthy controls.

Additionally, as with most human studies, a number of uncontrollable variables could potentially impact these results. Our study originally defined “On-HAART” as having been on stable HIV antiretroviral therapy for 6 months prior to the pre-HCV combination therapy biopsy; subject COINF 52 was “On-HAART” for 5 months and 3 days at the time of his pre-HCV combination therapy biopsy, and subject COINF 16 began HAART during the course of HCV combination therapy.
Although our study’s inclusion criteria excluded subjects who had other liver diseases, including infection with Hepatitis B virus, subject COINF 25’s chart indicated an infection with Hepatitis B between HCV combination therapy and the post-HCV combination therapy biopsy, along with continuing IDU (heroin and injected cocaine). In addition, COINF 25 had a previous infection of Hepatitis B before 2005 which was treated and resolved prior to his enrollment in this study. Despite this, this subject’s data falls within the ranges of the other subjects and did not constitute an obvious outlier.

Subject COINF 03, one of the earliest study participants enrolled, had conflicting information in his chart regarding his start date for HCV combination therapy, and it was therefore difficult to accurately ascertain the duration of his treatment, or if he received two overlapping rounds of treatment to extend therapy from 24 weeks to 48 weeks, which is the current treatment guideline for co-infected individuals regardless of HCV genotype. In 2004, when the subject enrolled in the study, the treatment guidelines stipulated 24 weeks of HCV combination therapy for patients infected with HCV genotype 3, regardless of their HIV status.

Also in regards to the exclusion criteria for this study, it was learned that subject COINF 24 was taking didanosine, a d-drug, as part of her HAART regimen at the time of pre-treatment biopsy despite the use of didanosine being an excluding criterion. Unfortunately, this was not discovered until after the subject had been enrolled in the study and had undergone her two liver biopsies for the purposes of this study.
5.2.3 Other Ultrastructural Analyses

When assessing cristae density by TEM, lighter electron density of matrices made identification of cristae membranes easier. When matrices were excessively dark, cristae membranes were more difficult to identify. These variables could potentially affect the accuracy of our estimates and introduce variability from sample to sample.

In this study, we did not select for or seek to identify cells in a particular stage of the cell cycle. The cristae and the inner mitochondrial membranes play a central role in the metabolic functioning of the mitochondria. Sweet et al. [128] examined the changes in mitochondrial membranes in a cell line of human leukemic cells throughout the cell cycle, noting that at different times in the cell cycle, inner mitochondrial membrane mass increased. Although they did not find a concomitant increase in ATP production with increase in mitochondrial membrane mass, they did note that before the start of G2 phase, 95% of the inner mitochondrial membrane growth had occurred. Posakony et al. examined the outer mitochondrial membrane contour length in HeLa cells at various points in the cell cycle, noting that the growth of the outer mitochondrial membrane grew proportionally to the cytoplasm throughout the cell cycle, as did the total mitochondrial volume fraction [129].

In our observations, we did not see mitotic hepatocytes in any of the biopsies. If mitosis followed a circadian rhythm then our collection of the biopsies at the same time of day following overnight fasting would have minimized any effect. There was also no significant difference in cell size before and after HCV combination therapy. Taken together, our findings do not suggest that the cell cycle of the hepatocytes biased our data.
5.3 Future Directions

The goal of this study was to establish the effect of HCV combination therapy on liver toxicity in HIV/HCV co-infected patients by examining hepatocyte ultrastructure. This study revealed interesting and significant results regarding liver pathology and hepatocyte lipid content following HCV combination therapy. A larger study with a greater number of participants would most likely help to increase confidence in our results. As treatment regimens and guidelines for HCV continue to evolve, it will become increasingly important to fully establish the combined effects of HAART, ribavirin and pegylated-interferon alpha on the liver of co-infected patients, particularly now with the recent addition of the proteases boceprevir and telaprevir in the treatment of HCV genotype I infections.

Taken together, our findings suggest that lipid metabolism in HIV/HCV co-infected hepatocytes and the reparative responses of the co-infected liver merit further investigation.
6. Conclusions

The use of new and improved drugs has dramatically improved disease outcome in patients co-infected with HIV and HCV. Despite this, liver toxicity remains a major concern, particularly as drugs for both infections are increasingly used simultaneously in co-infected patients. In the present study, we provide evidence that HCV combination therapy results in an overall improvement in liver pathology when given alongside HAART. In addition, liver steatosis as seen by hepatocyte lipid content is reduced following treatment. A number of other parameters were identified that are also likely to be affected by HCV combination therapy however increased sample size will be required to confirm these trends.

Taken together, the results of this study suggests that HCV combination therapy does affect hepatocyte ultrastructure while providing an overall beneficial effect to liver health in co-infected individuals also undergoing HAART. Future work investigating hepatocyte ultrastructure from a greater sized patient population will help determine additional ultrastrucutral changes resulting from HCV combination therapy.
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