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Impact of Highly Active Antiretroviral Therapy on Hepatitis C Virus RNA Levels over One Year in HIV-HCV Co-Infected Individuals

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Glossary

AIDS- acquired immunodeficiency syndrome; the name applied to HIV infection once a major opportunistic illness has occurred

Antibody- an immunoglobulin molecule that reacts with a specific antigen

Antigen- a molecule that stimulates an immune response

Antiretroviral Therapy- medical therapy directed at the suppression of HIV replication

CTL (Cytotoxic T Lymphocyte)-immune lymphocyte bearing a CD8 receptor and responsible for cellular immune response to antigen

CD4 T Lymphocyte- a lymphocyte, also known as a T helper cell, generally responsible for triggering immune responses to foreign antigens. Selectively depleted by HIV.

HAART- Highly Active Antiretroviral Therapy. A combination of at least three antiretroviral drugs from at least two different antiretroviral classes.

HCV RNA Level- The number of HCV virions, or genome copies, measured in copies per milliliter. By convention, changes in HCV RNA level over time are expressed as $\log_{10}$ HCV RNA copies/ml.

HIV RNA Level- The number of HIV virions, or genome copies, measured in copies per milliliter. By convention, changes in HIV RNA level over time are expressed as $\log_{10}$ HIV RNA copies/ml.

IVDU-intravenous drug use

Lymphocyte- a type of white blood cell involved in specific immune responses

Non-Nucleoside Reverse Transcriptase Inhibitor- also known as NNRTI; a drug that non-competitively inhibits HIV reverse transcriptase inhibitor

Nucleoside Reverse Transcriptase Inhibitor- also known as RTI or NRTI; drugs that competitively inhibit reverse transcriptase

Protease Inhibitor- also known as a protease inhibitor; drugs that inhibit HIV protease, which is required for post-translational cutting of HIV proteins

Reverse Transcriptase- the viral enzyme responsible for transcribing HIV RNA to DNA, which is then integrated into the cell genome

Abstract

The effect of highly active antiretroviral therapy (HAART) on plasma HCV RNA level in HIV-HCV co-infected individuals is uncertain. This issue was investigated over 12 months in HIV-HCV co-infected subjects HAART-treated for at least six months and achieving HIV RNA suppression below 500 copies/mL. It was predicted that HCV RNA would initially increase from baseline, decline thereafter, and fall below baseline by 12 months. Frozen plasma specimens were used to measure quantitative HCV RNA levels in 50 HAART-treated co-infected subjects at baseline, 3, 6, and 12 months. A 0.5 $\log_{10}$ increase in HCV RNA at 3 months was observed. This was followed by a decline below baseline. As low HCV RNA is a predictor of virologic response to HCV therapy, it may be advantageous to first achieve suppression of plasma HIV RNA level, gain immune reconstitution with HAART, and then initiate HCV antiviral treatment in HIV-HCV co-infected individuals.

1.0 Introduction

1.1 Background

The prevalence of hepatitis C virus (HCV) infection within the North American population is between 0.5% and 1.0% (1-6). Within several groups including hemophiliacs, intravenous drug users, prison inmates, certain ethnic populations, and HIV-seropositive individuals HCV prevalence is much higher (6-10). Approximately one-third of HIV-infected individuals are HCV seropositive (11). This rate is as high as 90% among intravenous drug users with HIV (2,6,7,9). HIV co-infection accelerates HCV-related fibrosis of the liver (12-15) and shortens the time period prior to the appearance of clinically evident liver disease (16-18). Mortality in HIV-infected subjects is also increased as a result of HCV-induced liver disease (18). Accelerated HCV disease in HIV-HCV co-infected individuals may result in part from impaired HCV-specific cell mediated immunity (19). The immunologic deterioration accompanying HIV infection may be halted, and in many cases partially reversed with potent and durable antiretroviral therapy. Thus treatment and improvement of HIV associated immune deficiency may be important to the progression and treatment of HCV infection and hepatitis. This is a reasonable suggestion, considering that immunologic improvement with effective highly active antiretroviral therapy (HAART) has been reported to result in reduction or resolution of co-infection with hepatitis B virus (20), hepatitis G virus (21), and human herpes virus 8 (22,23).

The relationship between HIV infection, immunodeficiency, and HCV RNA level are not understood. This is particularly true when evaluating the effect of HAART on this

interaction. This thesis evaluates the effect of HAART on HCV RNA level over a 12-month period in a select cohort of HIV-HCV co-infected individuals, with maximal therapeutic HIV RNA suppression.

1.2 Rationale

Change in HCV RNA level following the initiation of HAART in HIV-HCV co-infected individuals was observed in this retrospective, longitudinal cohort study. The current literature evaluating this is inconclusive. There are several reasons for this including small numbers of study subjects, inadequate statistical power, heterogeneous population samples, and limited duration of follow-up (see Introduction, Section 1). In evaluation of existing data, a biphasic response is predicted with an initial increase in HCV RNA level followed by a decline to below pre-HAART HCV RNA level.

A cohort of all HIV-HCV co-infected subjects on HAART followed at The Ottawa Hospital Immunodeficiency Clinic was assessed. The select nature of this cohort is a reflection of the specific question being asked in this study. The uncertainty in the literature was addressed by the provision of adequate power, and its inclusiveness despite specific criteria.

The data gathered from this study further defines the relationship between HIV, its successful treatment, and HCV over a prolonged period. As response to HCV antiviral therapy is more likely in those with low HCV RNA levels, the findings of this study may have relevant clinical implications. The timing of this intervention (i.e. prior to or

following HIV virologic suppression with HAART) is still a matter of debate. A clear demonstration of HCV RNA reduction with HAART would support the practice of treating HIV prior to HCV. Optimal management of HCV in co-infected individuals is important considering the significant morbidity and mortality due to HCV infection in HIV-seropositive individuals.

1.3 Objectives

Primary

- To evaluate the effect of the initiation of HAART on HCV RNA levels over the initial 12 months of therapy in HIV-HCV co-infected subjects.

Secondary

- To determine if certain key factors* influence baseline HCV RNA levels in HIV-HCV co-infected subjects.

- To determine if certain key factors* influence the size or direction of change in HCV RNA from baseline in HIV-HCV co-infected subjects initiation HAART.

*These factors include sex, age, mode of HCV infection, CD4 and CD8 T lymphocyte count, HIV RNA level, class of antiretroviral used for therapy, and chronic hepatitis B infection-status. These are standard variables evaluated in all antiretroviral studies of HIV-HCV co-infected individuals. Alcohol consumption was also evaluated, as it is known to influence HCV RNA levels in HCV mono-infected individuals.

1.4 Research Questions Pertaining to Primary Objective

1.4.1 Primary Research Question

What is the effect of HAART on HCV RNA level?

The effect of HAART on HCV RNA level is uncertain. It may be important to know as HCV RNA is correlated with the likelihood of response to HCV antiviral drug therapy.

Primary Hypothesis to be refuted

- At six months following the introduction of HAART, HCV RNA level will fall below baseline levels by a minimum of 0.5 $\log_{10}$.

1.4.2 Secondary Research Question #1

What is the effect of HAART on HCV RNA level early following the initiation of therapy?

The early effect of HAART on HCV RNA level is uncertain but most literature suggests an initial increase. This may be important in explaining hepatotoxicity in HIV-HCV co-infected subjects, which is often observed early following initiation of HAART.

Secondary Hypotheses to be refuted

- At three months following the introduction of HAART, HCV RNA level will transiently increase above baseline.

1.4.3 Secondary Research Question #2

What is the effect of HAART on HCV RNA level once HAART is well established?

Current literature is unclear as to the effect of long-term, virologically active HAART on HCV RNA levels.

Secondary Hypotheses to be refuted

- The decline in HCV RNA level seen at six months will be sustained at twelve months.

1.5 Literature Review

Section One:

A Review of the Effect of Highly Active Antiretroviral Therapy on HCV RNA Levels in HIV-HCV Co-Infection

Introduction

Despite the frequency of HIV-HCV co-infection, the relationship between HIV infection, immunodeficiency, and HCV RNA levels is not well understood. This is particularly true when evaluating the effect of highly active antiretroviral therapy (HAART) on this relationship. Greater understanding is warranted given that liver fibrosis is accelerated (12-15), time prior to the appearance of clinically evident liver disease is shortened (16-18), and mortality in HIV-infected subjects is increased as a result of HCV-induced liver disease in HIV-HCV co-infected subjects (18). In an effort to achieve clarity, a Medline search of English language journals using the key words HCV, HIV, viremia, and antiretroviral were used to identify relevant articles. References in identified papers were assessed for additional pertinent publications and conference abstracts. Abstracts from recent HIV and Infectious Diseases meetings (24-31) were evaluated. This review describes the apparent effect of antiretroviral therapy on HCV RNA levels from an analysis of available information, evaluates the strengths and weaknesses of these papers, and suggests a possible explanation for the findings reported in these studies. The relevance to HCV and HIV therapy in HIV-HCV co-infected individuals is considered.

Early Effect of HAART on HCV RNA Levels

The early response of HCV RNA levels to HAART remains controversial (Table 1). Having considered the potential influences on HCV RNA levels measurement in HIV-HCV co-infected subjects, the effect of HAART on HCV RNA levels can be better evaluated and interpreted. Several studies suggest that HCV RNA levels may initially increase marginally in antiretroviral-treated subjects (32-35). Puoti *et al* described a $\geq$0.2 $\log_{10}$ increase in 10 of 12 HAART-treated patients at 14 and 21 days. No difference was found between therapies consisting of protease inhibitor [indinavir (n=6)] or non-nucleoside reverse transcriptase inhibitor [nevirapine (n=6)] based treatment (32). This sample size is insufficient to identify a difference, even if present. Vento *et al* identified a mean HCV RNA levels increase of 0.48 $\log_{10}$ at one month in 51 individuals initiating indinavir or ritonavir-based treatment (33). By month three however, the change from baseline was less than 0.3 $\log_{10}$. In this study, the HIV plasma viremia declined by a mean of 1.68 $\log_{10}$ over the first month however maximal viral load suppression, defined as HIV RNA below 50 copies/mL, was not achieved in the majority of these subjects by three months. It is uncertain whether a larger increase in HCV RNA levels would have occurred if HIV RNA had been maximally suppressed. Rutschmann *et al* reported an overall mean 0.37$\pm$0.13 $\log_{10}$ increase in HCV RNA from a baseline of 5.27$\pm$0.22 $\log_{10}$, and >0.5 $\log_{10}$ increase in 9 of 19 HAART-treated subjects at 6 weeks (34). Once again, HIV RNA was not maximally suppressed. An increase in HCV RNA levels shortly after the introduction of HAART has not been universally reported (36-39). These

discrepancies between short term studies may be explained by a number of factors including different sampling times, variable HIV RNA suppression, variable duration and stages of HIV infection and immunologic status, differences between HCV assay sensitivities and accuracy, patient adherence to treatment, and antiretroviral hepatotoxicity. Although a meta-analysis might provide further insight, the above issues, indicative of the heterogeneity of these study groups and lack of their characterization, make such an exercise inappropriate.

A pronounced clinical manifestation of this initial increase in HCV RNA levels, associated with transaminitis, signs of tender hepatomegaly, and occasionally death, is observed with the so-called 'immune restoration syndrome' which has been described following introduction of HAART in HIV-HCV co-infected individuals (40-42). The pathophysiologic explanation of the early rise in HCV RNA levels reported with this syndrome and immediately following the initiation of HAART may be cytotoxic T lymphocyte mediated lysis of HCV-infected cells, with resulting serum alanine aminotransferase elevation and release of HCV to the plasma (34,40,43). Other possible explanations include reduced HIV competition for CD81+ cell receptor, a suspected HCV cell entry receptor, thereby allowing for increased infection and replication by HCV and/or reduced HIV-induced endogenous interferon-α, with a resultant transient increase in HCV RNA levels (32,44). It is also possible that antiretroviral-specific hepatotoxicity may be responsible for cellular injury with resulting release of HCV RNA however the liver histopathology of these reported cases are inconsistent with direct drug-induced hepatotoxicity (33,40).

HCV genotype could theoretically influence HCV RNA levels following the initiation of HAART (32,39). Puoti *et al* reported the HCV genotypes of his study subjects but the number of individuals assessed (n=12) was insufficient to draw any meaningful conclusions (32). Furthermore, 8 subjects were infected with genotype 3a, and 4 with genotype 4c/4d, which are common in Italy but found less frequently in other parts of the world including North America. A study with sufficient number of patients and mix of HCV genotypes is required to determine if HCV genotype is a relevant factor in predicting change in HCV RNA levels following HAART therapy.

There is evidence that interferon in combination with nucleoside reverse transcriptase inhibitors is virologically active against HIV (45,46). In contrast, there is no evidence to suggest that either nucleoside reverse transcriptase inhibitors or protease inhibitors possess direct anti-HCV activity (32-38). There are few data assessing non-nucleoside reverse transcriptase inhibitor anti-HCV activity, and none that is conclusive (32). Although underpowered, the Puoti *et al* study suggested no difference in the direction or degree of HCV RNA levels change between protease inhibitor and non-nucleoside reverse transcriptase inhibitor-based therapy (32). Although further investigation would be ideal, the type of antiretrovirals used is unlikely to have a significant direct effect.

Table 1

Effect of HAART on HCV RNA Levels in the Initial Three Months

Publication (reference)	N	Antiretroviral Therapy	Baseline CD4 (cells/μL)	Baseline HCV (log_{10})	Month 1-2 HCV (log_{10})	Direction Change Month 1-2**	Month 3 HCV (log_{10})	Direction of HCV Month 3**
Rutschman (34)*	19	PI plus 2 nRTI	63±13	5.27±0.22	Δ.37±0.13	⇑ (p=0.01)	NR	NR
Puoti (32) *	12	IDVor NVP, 3TC,D4T	338±93	7.0±0.2	Δ .03± 0.1	⇑ (p=NS)	Δ -0.18± 0.16	⇓ (p=NS)
Matsiota-Bernard (39)*	10	PI plus 2 nRTI	84±57	4.59±0.53	Δ nil	NC	Δ nil	NC
Vento (33)	21	IDV/3TC/ZDV	316	5.54	6.05	⇑	5.81	⇑
	11	IDV/3TC/D4T	332	5.58	6.02	⇑	5.87	⇑
	19	RTV/ZDV/3TC	325	5.52	5.99	⇑	5.81	⇑
Zylberberg (37)	22	PI plus 2 nRTI	119±118	5.5±0.8	NR	NR	5.3±0.7	⇓ (p=NS)
Rockstroh (47)	13	SQV,3TC, ZDV or D4T	179±139	6.56±6.04	NR	NR	6.46±5.86	⇓ (p=NS)
	13	IDV,3TC,ZDV or D4T	38±41	6.54±5.95	NR	NR	6.52±5.95	⇓ (p=NS)
Perez-Olmeda (36)	16	PI plus 2 nRTI	NR	~7	NR	NR	~7	NC (p=NS)

* mean change in viral load reported as opposed to change in group mean
** P value stated were reported
⇑ increase in HCV RNA
⇓ decrease in HCV RNA
NC no change in HCV RNA
NR not reported
NS not significant
PI protease inhibitor
NRTI nucleoside reverse transcriptase inhibitor
IDV Indinavir
NVP Nevirapine
RTV Ritonavir
SQV Saquinavir

Effect of Prolonged HAART on HCV RNA Levels

CD4+ T cell counts and functional immune competence generally increase as HIV RNA falls following the introduction of HAART (48-50). Despite several studies addressing this issue, it is unclear whether the decline in HIV RNA and increase in CD4+ T lymphocyte count, a measure of immune restoration, are associated with a reduction, an increase, or no change in HCV RNA levels (Table 2). At least one study described a reduction in HCV RNA levels by greater than 0.5 $\log_{10}$ at 12 months treatment in 7 of 16 patients on HAART with HIV RNA suppressed to below 50 copies/ μl (36). This in fact is the only long-term evaluation of HCV RNA levels in which HIV RNA was maximally suppressed with antiretroviral therapy. The mean CD4+ T lymphocyte increase at 12 months was 210±18 cells/ μL, indicative of significant immune restoration. In this cohort, HCV RNA levels fell below the limit of detection (100-1000 HCV RNA copies/ml) in 4 of 16 subjects. This has been reported elsewhere in 2 of 31 (51) and 2 of 10 (35) subjects following the initiation of HAART, however most other studies suggest that clearance of HCV is not usual in HIV-HCV co-infected subjects. Nonetheless, these results suggest that prolonged suppression of HIV replication associated with immunologic restoration may result in improved immune control of HCV infection. Yokozaki *et al* assessed twenty-five HIV-HCV co-infected subjects at baseline and at six and twelve months following the introduction of HAART (51). At baseline, the mean HCV RNA levels was approximately 9 x 10^6 genome equivalents per milliliter (Eq/ml) by branched DNA probe (Quantiplex HCV RNA, Chiron). By 6 months, the mean HCV RNA levels had fallen to approximately 6 x 10^6 Eq/ml and approximately 4 x 10^6 Eq/ml by 12 months. When

transformed to $\log_{10}$ values, this reflects a change of <0.5 $\log_{10}$ Eq/ml. The biological relevance of this is unclear. This study is limited by the failure to report exact values and by the reporting of results as a group mean as opposed to by the degree and direction of change for each individual. Furthermore, the HIV assay lower limit of detection was 400 HIV RNA copies/mL, a measure that by current standards of measurement and treatment. is not maximally suppressed.

Other studies assessing the long-term effect of HAART on HCV RNA levels with data at three (32,47), four (34), six (39,52), nine months (37), 12 months (53), and 24 months (54) following the start of HAART have not duplicated the above findings. In a study of 24 months, Torre *et al* reported a small, statistically insignificant change in HCV RNA levels in 65 HIV-HCV co-infected subjects on HAART. Furthermore, change in HCV plasma viremia was evaluated as a function of baseline alanine aminotransferase level and on whether immune restoration, defined as an increase in CD4 T cell count from ≤ 200 cells/μL at baseline to ≥ 400 cells/μL at 12 and 24 months, was achieved (54). These parameters did not appear to influence the effect of long term HAART on HCV RNA. As in other studies, HIV RNA was not maximally suppressed and a subgroup analysis of those achieving maximal HIV virologic suppression was not performed. These studies are limited by the fact that they report only the changes in group mean over time and do not comment on the amount and direction of change for individuals. Furthermore, in several of these studies, follow-up for all individuals contributing to the baseline group mean HCV RNA levels measurement is not complete (34,37,54) or not clearly stated (53) thereby making comparison between HCV RNA levels at baseline and time points

thereafter problematic. It should not be assumed that the baseline level of HCV RNA or the degree and direction of change in this measure following the initiation of HAART was the same for those with complete follow-up measurements and for those without. An additional concern is that most of these studies only assess two time points, one at baseline and one other subsequent measure. Ideally, multiple time points are required in order to detect and measure a relationship between progressive immune restoration from HAART therapy and HCV RNA levels.

Further confusing this issue are two studies describing an increase in HCV RNA levels at 48 and 96 weeks of HAART (52,55). In these studies the change in HCV RNA levels described was less than 0.3 $\log_{10}$, a magnitude of change explicable by biological and systematic variability. As well, this minimal change in HCV RNA levels is of uncertain biological relevance. A significant problem with each of these studies is that HIV RNA was not maximally suppressed (i.e. <50 HIV RNA copies/ml), and for this reason it can be argued that optimal immune restoration was not achieved. These studies are small and underpowered. Once again, mean group HCV RNA levels was reported but a clear description of within subject variability of this measure over time is lacking and follow-up is not complete.

An important paper recently published suggests that liver HCV RNA level was approximately three-fold lower in subjects receiving at least 6 months of protease inhibitor-based HAART compared to those not on HAART (56). In contrast, the serum HCV RNA levels did not differ significantly in this study. This paper suggests that serum

HCV RNA measurement may not be highly correlated with those within the liver. It also suggests that long-term HAART treatment results in a reduction in HCV RNA levels. Further evaluation of liver HCV RNA level following the initiation of HAART is warranted. Liver biopsies are required in order to quantitate liver HCV RNA levels. Given the risk and discomfort involved with liver biopsies, serum HCV RNA quantitation will continue to be a necessary tool used to evaluate the effect of HAART on HCV RNA infection.

Table 2

Long-Term Effect of HAART on HCV RNA Levels

Publication (reference)	N	Antiretroviral Therapy	Baseline CD4 (cells/μL)	Baseline HCV (log_{10})	Month 4-6 HCV (log_{10})	Month 9-12 HCV (log_{10})	Direction of HCV Change	P value
Rutchmann (34)*	19	PI ± nucleoside(s)	63±13	5.27±0.22	Δ-0.16 ±0.23	Δ-0.14 ±0.19	⇓	NS
Matsiota-Bernard (39)*	10	PI plus 2 nucleoside	84±57	4.59±0.53	Δ NC	NR	NC	NR
Zylberberg (41)	22	PI plus 2 nucleoside	119±118	5.5±0.8	5.3±0.3	5.3±0.3	⇓	NS
Perez-Olmeda (36)	16	PI plus 2 nucleoside	NR	~7	NR	~6	⇓	NR
Yokozaki (51)	25	PI plus 2 nucleoside	~325	~6.95	~6.78	~6.6	⇓	<0.01
Ragni (52)	21	PI plus 2 nucleoside	152	7.15	7.05	7.30	⇑	<0.05
Bush (57)	21	HAART	NR	5.78	5.81	NR	⇑	NR
Gavazzi (53)	10	PI plus 2 nucleoside	172±121	5.28±0.26	NR	5.39±0.26	⇑	NS
	12	PI plus 2 nucleoside	202±98	4.65±0.32	NR	4.82±0.33	⇑	NS
Vento (33)	21	IDV/3TC/AZT	316	5.54	NR	5.66	⇑	NR
	11	IDV/3TC/D4T	332	5.58	NR	5.71	⇑	NR
	19	RTV/AZT/3TC	325	5.52	NR	5.69	⇑	NR
Torre (54)	65	HAART	NR	6.53	NR	6.63**	⇑	NS

* mean change in viral load reported as opposed to change in group mean

** 24 month data

⇑ increase in HCV RNA
⇓ decrease in HCV RNA
NC no change in HCV RNA
NS not significant
NR not reported
PI protease inhibitors
HAART highly active antiretroviral therapy
IDV indinavir
RTV ritonavir

The Effect of Discontinuation of HAART on HCV RNA Levels

An interesting but minimally explored question is the effect of HAART discontinuation or virologic HIV treatment failure on HCV RNA levels. Bush *et al* described a cohort of twenty-one HIV-HCV co-infected subjects with median HIV RNA below 400 copies/ml four months after the initiation of HAART (57). Eight of these subjects subsequently failed antiretroviral therapy. A median increase in HCV RNA levels to 6.3 $\log_{10}$ copies/ml was reported in these eight individuals from 5.78 $\log_{10}$ copies/ml at baseline and 5.81 $\log_{10}$ copies/ml at four months. This issue merits further exploration.

Clinical Relevance of HCV RNA Levels in HIV-HCV Co-Infection

If present, a reduction in HCV RNA levels as a result of initial HIV therapy could justify deferral of HCV treatment in co-infected individuals for two reasons. There appears to be a continuous relationship between HCV RNA level and likelihood of sustained response to interferon and ribavirin antiviral therapy, defined as plasma HCV RNA level negativity six months following completion of treatment (58-61). In subjects with HCV RNA levels greater than 2.0×10^6 HCV RNA copies/ml by National Genetics Institute HCV SuperQuant system (equivalent to about 2.0×10^5 HCV RNA copies/ml by the Roche AMPLICOR HCV Monitor system), the sustained response following 48 weeks of interferon and ribavirin was 36% compared to 43% for those with HCV RNA levels below this level (59). The importance of HCV RNA level has been demonstrated in other

studies as well (61). It is reasonable to assume that the same is true in HIV-HCV co-infected subjects. If HCV RNA level is indirectly reduced by potent and durable antiretroviral treatment, then the likelihood of responding to interferon and ribavirin therapy with sustained clearance of HCV RNA levels may be increased. If this assumption is demonstrated to be true, then given incomplete efficacy of current HCV treatment, this would provide a strong rationale for the delay of HCV antiviral therapy until after effective HIV antiretroviral therapy was established.

In immune competent hosts, HCV RNA levels varies greatly between individuals with HCV infection and, in most reports, does not correlate with or predict the progression of liver disease (59,61). It is uncertain whether the same is true in immune compromised. HIV-HCV co-infected individuals. As HCV RNA levels are higher and liver fibrosis occurs more rapidly in HIV-HCV co-infected subjects, it is plausible that there is a correlation between HCV RNA level and progression of liver disease in this population. A retrospective study assessing liver fibrosis scores before and a median of 14 months after protease inhibitor-based antiretroviral therapy in HIV-HCV co-infected subjects suggests that the rate of fibrosis progression was significantly slower in patients treated with protease inhibitor and nucleoside reverse transcriptase inhibitors compared to those treated with nucleoside reverse transcriptase inhibitors only (62). It is unclear whether these findings were a result of an indirect HAART-induced decline in HCV RNA levels. improved HCV-specific immune response, a direct protease inhibitor effect on fibrosis matrix synthesis, or a combination of these factors.

Conclusion

Although several studies attempt to describe the natural history of HCV RNA levels following the initiation of HAART, no single report clearly describes it from the introduction of therapy to a point when antiretroviral activity is well established and immune reconstitution is equilibrated. Many of these investigations are of small size thereby limiting the ability to draw meaningful conclusions. Almost all results are based on subjects without maximal therapeutic suppression of HIV RNA. The effect that discontinuing or failing HAART may have on HCV RNA levels is uncertain. A critical review of the literature demonstrates that the natural history of HCV RNA levels in HAART-treated, HIV-HCV co-infected individuals remains to be fully defined. A well-designed, prospective study of sufficient power and duration is needed to clarify these issues.

Section Two:

Measurement of HCV RNA Level –Systematic and Biologic Variation

There are multiple factors, which may influence the results obtained by single and serial measurement of HCV RNA in HIV-HCV co-infected subjects. It is important to be aware of the issues determining the accuracy and variability of HCV RNA measurement, review the characteristics of HCV RNA in HIV-HCV co-infected individuals, and evaluate those factors, which may affect this measure.

The systematic variation in within-run and between-run precision of the quantitative assay used to determine HCV RNA levels [Cobas Amplicor HCV MonitorTM Test. Version 2 (Roche Diagnostics Systems, Inc.), branched DNA 2.0 system (Chiron), nucleic acid sequence-based amplification (NASBA) (Organon) assay] has not been evaluated specifically in HIV-HCV co-infected cohorts. There is ample data evaluating this in HCV-infected subjects, however (63-69). The within-run and between-run precision of the assays utilized in the literature are comparable (65,66,69). There is good concordance between the branched DNA and reverse transcriptase polymerase chain reaction systems of quantifying HCV RNA levels (R^2=0.745) (69).

Most studies report results on specimens in frozen storage for months or even years. Halfon *et al* reported a 10% decline in HCV RNA levels stored at -80°C for six months (70). Other studies suggest that the HCV RNA levels remained stable while in varying periods of long-term frozen storage, assuming appropriate prestorage handling (71,72).

Krajden *et al* demonstrated that multiple freeze-thaw had a small but clinically insignificant effect on HCV RNA levels (73).

Having considered sources of systematic variation in HCV RNA measurement, the clinician should evaluate those sources which are biologic. Although the magnitude may not be fully resolved, it is well documented that HCV RNA level is higher in HIV-HCV co-infected individuals than in HIV-seronegative cohorts (16,51,74-79). Cribier *et al* compared 59 HIV-HCV co-infected to 51 HCV-infected subjects and demonstrated a greater than 0.5 $\log_{10}$ higher HCV RNA level in the former (77). Similar results were reported in a group of 343 HIV-HCV co-infected hemophiliacs in comparison to 42 HCV-seropositive but HIV-seronegative hemophiliacs (76). In a study controlling for age, HIV RNA level, and CD4+ T lymphocyte count Dragoni *et al* also demonstrated higher HCV RNA level in HIV-HCV co-infection (16). These studies are limited by the fact that antiretroviral therapy, a factor that might influence HCV RNA levels, is not fully described or controlled for in multivariate analysis.

HCV RNA level is relatively stable over time in chronically infected individuals not receiving HCV antiviral therapy (52,64,70,80-86). Over a period of two to three months HCV RNA level fluctuates by no more than 0.75 $\log_{10}$ (70,85). Arase *et al*, in a study of 212 subjects reported HCV RNA level fluctuations of less than 1.0 $\log_{10}$ in 93% of subjects over a two-year period (84). These results may not be generalizable to all HCV-infected populations. Only those infected with genotype 1b (1694 of 2248; 75.4%) and with screening HCV RNA levels greater than 1.0×10^7 mEq/ml (319 of 1694; 18.8%) by

Chiron Quantiplex branched DNA assay were included in this analysis. Furthermore, only subjects with monthly HCV RNA level measures over two years and without antiviral or immunosuppressant use during the study period (212 of 319; 66.5%) were included in this evaluation. In a cohort of 14 followed for a range of 3.7 to 6.6 years, HCV RNA level fluctuated by less than 1.0 $\log_{10}$ in all but one individual (81). In a study of 60 followed for a median of 40.4 months, the group mean HCV RNA level fluctuated by greater than 1.5 $\log_{10}$ (83). In this study, levels varied over time by less than 1.0 $\log_{10}$ in 62% of patients, 1.0-1.5 $\log_{10}$ in 22% and greater than 1.5 $\log_{10}$ in 17%. The reason for greater fluctuation of HCV RNA level in this cohort is unclear. Each of the above studies utilized the Chiron Quantiplex branched DNA assay system.

The stability of HCV RNA level is less well described in HIV-HCV co-infected subjects (74,80,82). In HIV-HCV co-infection, HCV RNA level stability, defined as group mean HCV RNA level fluctuations of less than 1.0 $\log_{10}$, was reported over the initial two to five years of infection in a cohort of thirteen individuals (74). This was likewise observed in four HCV-HIV co-infected individuals followed for over two years (80). These subjects were not on antiretroviral therapy. Over a mean follow-up period of seven years, HCV RNA level in 175 HIV-HCV co-infected subjects with minimal antiretroviral exposure was relatively stable with a baseline mean of 6.67 $\log_{10}$ copies/ml and a mean of 6.54 $\log_{10}$ copies/ml at seven years (82). Within group individual variation, as well as selective follow-up were not addressed thereby limiting the ability to interpret the results of this study. Keeping these limitations in mind, the best observations suggest HCV RNA level fluctuations do not exceed 1.5 $\log_{10}$ in HIV-HCV co-infected individuals.

The extent of immune deficiency, estimated by measurement of the absolute CD4+ T lymphocyte count, may influence HCV RNA level in HIV-HCV co-infection. An inverse correlation between absolute CD4+ T lymphocyte count and HCV RNA level has been described (74,82). Eyster *et al* reported relative stability in HCV RNA level until profound immune suppression developed, indicated by CD4+ T lymphocyte counts below 200 cells/μL (74). Daar *et al*, in a cross-sectional study, reported an inverse relationship between CD4+ T cell count and HCV RNA level (82). The 0.028 $\log_{10}$ copies/mL increase per 100 cells/uL change in CD4+ T cell count described in this paper is not clinically significance (82). Beld *et al* assessed nine HIV seropositive subjects without profound immune suppression and reported a very weak inverse relation (80). Several studies evaluating larger cohorts do not identify a correlation between CD4+ T lymphocyte count and HCV RNA level (16,51,78,87). Overall, these data suggest that HCV RNA level is relatively stable in HIV seropositive individuals, but that with advanced immune deficiency, as indicated by low CD4+ T lymphocyte count, some elevation in HCV RNA level may occur.

Alcoholism is a significant medical issue in many HIV-HCV co-infected individuals (88). Excessive consumption of alcohol, defined as greater than 50 grams per day (i.e. approximately three alcoholic beverages per day) may also increase HCV RNA level (52,89-91). This relationship appears to be linear (89). Although the mechanism is poorly understood, it is speculated that increased release of HCV RNA level from hepatocyte lysis, increased viral replication, and impaired HCV clearance resulting from alcohol-induced immune suppression may contribute to this phenomenon (92,93). Excess alcohol

may blunt immune restoration following the initiation of HAART (94) and/or accentuate antiretroviral-related hepatotoxicity with subsequent increase in HCV RNA level (95). These factors may influence the amount and direction of change in HCV RNA level following the initiation of antiretroviral therapy. Despite the importance of this issue, the literature evaluating HCV RNA level and its relationship to HAART does not address concomitant alcohol use.

Other factors potentially influencing HCV RNA level include severity of hepatocellular dysfunction, HIV RNA level, and HCV genotype (16,68,96,97). In immune competent hosts, HCV RNA level does not appear to differ significantly among individuals with chronic active hepatitis with or without compensated cirrhosis (96). An increase in HCV RNA levels in HIV-HCV co-infected patients with endstage liver disease has been described (74). This may be partially explained by lower CD4+ T lymphocyte counts in these individuals. The major cell receptors and target cells of these two viruses differ, therefore it is possible that HCV replication may be virologically independent of the level of HIV RNA level. A lack of correlation between HCV and HIV RNA level has been reported suggesting that this assumption is accurate (51,80). Both viruses may utilize CD81 cellular receptors. The relevance to the HIV and HCV RNA level is uncertain (98). As in HIV-seronegative individuals, HCV genotype does not appear to influence HCV RNA level in HIV-HCV co-infected individuals (68,75,77,96,97,99).

These investigations demonstrate that many factors including systematic variation of quantitative assays and natural or biologic variability of HCV RNA level must be

considered when evaluating HCV RNA level over time. For this reason, caution should be exercised when evaluating studies reporting mean HCV RNA level $\log_{10}$. This is analogous to HIV RNA levels measurements, as the sum of biologic and systematic variation is 0.5 $\log_{10}$ copies/mL according to Centers for Disease Control and Prevention treatment guidelines for HIV infection (100). Given the limited and problematic data currently available, it is difficult to determine a similar value for HCV RNA level with great confidence. Nonetheless, taking biologic HCV RNA level variability and assay variability into consideration, a change of at least 0.5 $\log_{10}$ is more likely to be of biological, if not clinical significance.

Section Three:

Issues Pertaining to Study Design

The effect of HAART on HCV RNA levels in HIV-HCV co-infected individuals remains unclear despite the existence of many studies evaluating this issue (32-34,36,37,39,41,47,52,53,57). This is in part a result of small sample sizes. The time points in which HCV RNA levels were measured differ between studies which further compromises the ability to compare results (Tables 1, 2). These studies do not describe how subjects were selected for inclusion. In most cases, it appears as though samples of convenience were used for analysis. This limits the generalizability of the reported effect of HAART on HCV RNA level.

An objective of this study was to determine if initiation of HAART causes changes in HCV RNA level. Other studies have attempted to establish causation but lack of statistical power precludes this. Most reports of the early effects of HAART on HCV RNA levels consistently demonstrate an increase (Table 1). Inconsistency of results is a characteristic of longer-term studies (Table 2). This results primarily from heterogeneity of populations. The cohorts evaluated differ in terms of antiretroviral drugs utilized, degree of HIV RNA suppression, and pre-treatment immune status. The degree of HIV RNA suppression differs within and between studies and is poorly reported. This, in combination with small sample size precludes identification of a biological gradient, if present. The suggested mechanisms responsible for the initial increase and subsequent decline in HCV RNA level are biologically plausible. In some cases, liver biopsy and

biochemical data support these proposed mechanism (33). Most papers do not provide the laboratory data required to confirm or refute these hypotheses (32,34,37). There is little data evaluating the effect of HAART on HCV-specific immunity (101,102). Until this is better elucidated, the biologic plausibility and coherence of these explanations will remain in question.

A prospective evaluation of HIV-HCV co-infected subjects initiating HAART would be an ideal study design with which to assess the effect of HAART on HCV RNA levels. Randomization could be stratified for certain key parameters potentially influencing the degree and/or direction of HCV RNA level change from baseline. This design would address issues of selection bias as well (e.g. some clinicians reserve protease inhibitor use for individuals thought to have minimal HCV-related liver disease). Although it would be difficult to stratify for alcohol use, the amount of consumption would be quantitatively captured by standardized questionnaire. This would reduce the likelihood of misclassification. By a prospective study design, HIV RNA suppression in all study subjects would be unlikely therefore maximal immune restoration would not be achieved. At the very least, and in contrast to published studies, HIV RNA levels over the time of evaluation could be accurately captured. This would allow for the assessment of a biologic gradient between degree of HIV RNA suppression (with concurrent immune restoration) and change in HCV RNA level from baseline.

A retrospective evaluation of HIV-HCV co-infected subjects was utilized to address the relation between HAART and HCV RNA levels. This approach allowed for timely

completion of this study and made possible the study of a homogenous population by selecting for key parameters. The key issue of differential HIV RNA suppression, which limited other studies, was addressed by this study design. Only subjects with maximal virologic suppression were included in the final cohort of this study, thereby controlling for this parameter. Another key issue addressed by this study design was sample size. A prospective study of 50 to 75 HIV-HCV co-infected subjects would require several years to enroll and complete follow-up. In contrast, given the large volume of patients followed at The Ottawa Hospital Immunodeficiency Clinic, the rapid identification of a sufficiently sized cohort was possible.

Although problems including missing data and misclassification are more likely with retrospective studies, the accuracy and completeness of data collected in The Ottawa Hospital Immunodeficiency Clinic is acceptable. A small number of highly trained HIV-care providers enter patient information into the charts. Furthermore, standardized initial visit information sheets are utilized by clinic nurses. Because the available data on each subject is broad and accurate, several parameters which were not previously assessed (i.e. alcohol consumption, prior antiretroviral use, classes of antiretroviral drug used) could be assessed in this study.

Another strength of this study design is that loss to follow-up and patient dropout are not issues. HIV-HCV co-infected subjects frequently discontinue therapy within one year of initiation (103). If a prospective study design was used, approximately twice the number of subjects would need to be enrolled to ensure an adequate sample size to evaluate the

primary question of this study (i.e. What is the effect of HAART on HCV RNA levels following 6 months of therapy?).

A potential limitation of this study design is the absence of a control arm. A matched study design in which a control arm of HIV-HCV co-infected subjects not on HAART could have been created to demonstrate stability of HCV RNA level over time. The ability to match HIV-HCV co-infected subjects on HAART for at least six months with those not on HAART would be difficult given that the matching parameters (i.e. alcohol use, HBV status, IVDU, CD4 T lymphocyte count, HIV RNA level) are key factors influencing whether subjects start HAART. A review of the literature demonstrates the serial HCV RNA levels are stable over time (52,64,70,80-86). This is sufficient to preclude the absolute need for a control arm. Studies demonstrate minimal short-term variability in HCV RNA (65,66,69), therefore the HCV RNA level measured immediately prior to initiation of HAART was used as a baseline value to which subsequent HCV RNA measures were compared.

The evaluated cohort was selected from all HIV-HCV co-infected subjects followed at The Ottawa Hospital Immunodeficiency Clinic and therefore is more reflective of the HIV-HCV co-infected population in general, as opposed to the data obtained from samples of convenience reported in the literature.

In short, this study design addresses the key issues of sample size, statistical power, population heterogeneity, and generalizability, which influence the results of previous

studies. The results of this study are derived from a well defined and homogeneous population therefore interpretation and generalization to other HIV-HCV co-infected populations is possible and, furthermore, appropriate. This design is time efficient and not cost prohibitive. Any misclassification in this study was unlikely to be differential therefore the results of this analysis are unlikely to be overestimated.

Section Four:

Description of HCV and HIV Immunity

As articulated by Hill, biological plausibility is a key factor in determining the likelihood of causation (104). As HCV RNA level prior to HAART and following initiation of therapy is likely closely related to HCV-specific immune function, a brief description of HCV and HIV immunity is warranted.

HCV Immunity

HCV infection induces both humoral and cell-mediated responses to structural and non-structural HCV proteins. Despite this broad response only 10% to 15% of those exposed to HCV successfully clear the infection (105-107) (NB: although still debated, cleared refers to persistent serum HCV RNA level negativity and presumed elimination of HCV from other sites in the body). Although HCV-specific antibodies are generated and likely play a role in acquired immunity to HCV infection, the humoral response is insufficient to clear infection (108-117) or to prevent it (109). Cell-mediated immunity, and in particular, the cytotoxic CD8+ T lymphocyte (CTL) response appears pivotal (118,119).

HCV-specific antibodies are directed at both structural and non-structural proteins. Antibodies against non-structural proteins (NS-3, NS-4, NS-5) correlate with viral replication whereas core-specific antibodies are found in almost all exposed hosts, whether HCV has been cleared or not (110,111). Core-specific IgM antibody titers have been correlated with disease activity however, and decline with successful interferon-α

(IFN) therapy (112). Antibodies against envelop proteins are present in greater than 90% of patients (113,114) and those directed against the hypervariable region of E2 may play a role in early viral clearance through neutralization (115). Despite these observations, in general, the humoral response to HCV does not have adequate neutralizing effect to control infection (108,116,117).

CD8 cytotoxic T lymphocytes (CTL) and CD4 T helper lymphocytes appear to play significant roles in the control of HCV infection (118,119). Proliferative responses to several HCV-specific antigens have been described *in vitro* to both of these subsets of T lymphocytes (120-123). Additionally, both of these cell types are found in the inflammatory infiltrates of infected liver (124). In an effort to elucidate the role of CD4 T cells in HCV immunity, Cramp *et al* identified two cohorts (108). One group consisted of previous HCV-exposed individuals with positive HCV antibody titers but persistently negative HCV RNA measurements. The second group consisted of HCV antibody and RNA positive subjects. CD4 T lymphocyte proliferative response to at least one of four HCV antigens including NS3, NS4, NS5, and core was more frequently observed in the former group than the latter (23 of 35 versus 5 of 31). The production of IFN-γ, a Th1 cytokine, was significantly increased in the HCV RNA negative group in response to all four antigens tested but not in the HCV RNA positive cohort. The strength of CD4 T lymphocyte proliferative response to the HCV protein correlated closely with the magnitude of the increase in IFN-γ production to the same protein. Of note, no response to NS3, NS5, or core was noted in the HCV RNA positive group. These results suggest that individuals who are capable of mounting a strong Th1 response to HCV antigen may

be more likely to clear this virus and indicates that effective CD4 T lymphocyte activation plays a role in control of HCV replication.

Several studies provide evidence of HCV-specific CD8+ T cells role in HCV control (125-129). A strong, polyclonal CTL response in the peripheral blood and liver has been associated with lower HCV RNA levels (126,127). HCV-exposed individuals without history of antibody detection or transient viremia also exhibit polyclonal CTL (128,129). In addition to a direct cytotoxic effect against HCV infected cells, cytokine production by CD8 T lymphocytes is likely critical to effective HCV immune response. IFN-γ producing HCV-specific CD8 T cells were found most abundantly in patients with acute HCV who did not subsequently evolve to chronic infection (125). Although CD4+ T lymphocytes have a significant role in HCV immune response, HCV RNA persists despite the presence of HCV-specific CTL in many cases. Furthermore, considerable heterogenicity in detectable HCV-specific CTL responses in chronically infected individuals exists (130). These points help to illustrate that multiple factors influence the immune response to this infection.

The interplay between both cell-mediated and humoral immunity likely determines the natural history of HCV-induced infection in a given individual. A strong Th1 response with minimal Th2 response is generally observed in those individuals with acute HCV who do not progress to a chronic viremic state (131-135). The reverse is seen in chronically viremic subjects. Some have suggested that an enhanced Th2 / humoral immune response may actually prevent viral clearance based on these reports. Further

supporting this is the observed treatment benefit of IFN-α, a cytokine that down regulates Th2 response (135), in clearing chronic infection. Not all IFN-α responders have a predominant Th1 cytokine profile, however.

Given the roles that CD4 T cells, CD8 T cells, various cytokines, and humoral immunity play in controlling HCV RNA one can speculate that HIV infection, a process that profoundly effects each of these aspects of immunity, may effect the immune system's capacity to control HCV viremia. The following is a brief description of the impact HIV has on the immune system and the partial restoration of immune function achieved by HAART.

Immunologic dysfunction resulting from HIV infection

Impairment of innate, cell mediated and humoral immunity characterizes chronic HIV infection. As a result, susceptibility to opportunistic infections and malignancies is increased. Accelerated progression of HCV disease has been documented as well (12-18).

Alteration in CD4+ T cell number and function is the hallmark of HIV disease. Several studies have identified an inverse relation between CD4 T lymphocyte count and plasma HCV RNA level (74,80,82). CD4 T cell depletion may be due to a combination of impaired bone marrow production, impaired thymic maturation, and enhanced T cell death of both infected and uninfected cells due at least in part to increased apoptosis (136).

Polyclonal immune activation of both CD4 and CD8 T cells is observed in HIV infection, as indicated by increased proportion of cells, which express the activation markers CD38 and HLA-DR. Simultaneously, the frequency and proportion of naive peripheral T cells (which express CD45RA+, CD62L) is reduced (137,138). The pathogenic significance of immune activation in HIV disease may be demonstrated by observations that increased CD8T cell CD38 and HLA-DR expression is associated with a more rapid disease progression (139-141).

CTL activity, mediated by CD8+ lymphocytes, is important in host control of HIV (142-144). Effective CTL response is associated with reduced HIV RNA levels and slowed HIV disease progression (144,145). The loss of CTL directed against specific HIV-specific epitopes occurs with progression of HIV disease (146,147). Even in asymptomatic patients with CD4 T lymphocyte counts greater than 500 cells/uL the greater the degree of cell mediated immune dysfunction, the more likely the patient will progress to AIDS and death (148,149).

Another central feature of HIV infection is altered cytokine regulation. IL-2 and IL-12 are key cytokines in the generation of cell-mediated immunity. These cytokines are critical for CTL and NK cell proliferation and lytic activity, as well as other regulatory functions. These cell-mediated immune effectors represent the primary mechanism whereby most viral infections are cleared (150). In HIV infection the production of these cytokines by PBMC in response to various antigens is depressed (151,152). The balance between cytokines mediating Th1 type, cellular immunity (IL-2 and IFN-γ) and those

mediating Th2, humoral immunity (IL-4, IL-5, IL-6, IL-10) is altered in favor of the latter. This alteration in the helper cell compartment may be critical to the pathogenesis of HIV infection (153). Not only do these changes impair the generation of cellular immune responses, but also directly influence susceptibility of cells towards apoptosis. HIV-HCV co-infection has been reported to alter cytokine production response to in vitro HCV-specific antigen stimulation (154). Significantly higher IL-2 and INF-γ production was reported in HIV-HCV co-infected individuals in comparison to HIV or HCV infected subjects. Furthermore, HCV antigen stimulation of peripheral blood lymphocytes yielded increased production of IL-4, a Th2 cytokine, in the CD30 cells, a subset that is increase in HIV infected subjects. These results suggest that HCV may contribute both the immune activated state characteristic of HIV infection and contribute to the Th2 shift observed with HIV disease.

Immunologic restoration resulting from antiretroviral therapy

Effective antiretroviral therapy is associated with profound and diverse immunologic changes. There is a sustained increase in CD4T cell number which is inversely associated with a rapid drop in HIV RNA level. Several factors may contribute to the increase in CD4 T cell count including cellular redistribution (155), cellular proliferation of the peripheral T cell pool (156), new T cell synthesis from a thymic source (157,158), and reduction in apoptosis (159-165). Following the initiation of antiretroviral therapy in HIV infected subjects, the number of activated T cells decline (CD38 and HLA-DR) and the naive T cell population increases (CD4+CD45RA+CD62L+) (159,166-174). The mean expression of CD28+ on CD8 T lymphocytes increases to levels similar to

HIV-seronegative persons at 24 weeks following the initiation of highly potent antiretroviral therapy (166).

Cytokine dysregulation may be reversed by antiretroviral therapy; IL-12 production by peripheral blood mononuclear cells (PBMC) in response to some (LPS), but not all antigens (*Staphylococcus aureus*) is increased, as is PHA induced IL-2 production (166). IL-12 production is important in the generation of T helper cell and monocyte/macrophage function. IL-10 serum levels, which are increased in HIV-infected individuals and inhibitory to cellular immune response (175), are gradually reduced in patients receiving highly potent antiretroviral therapy (176). Following the introduction of HAART, plasma levels of the proinflammatory cytokine TNF-α are also reduced at 12 weeks, although not to those levels observed in HIV seronegative subjects (177). The magnitude of TNF-α reduction is associated with restoration of DTH response (177). Overall, the Th1 to Th2 shift that characterizes HIV infection is partially reversed by effective therapy however Th2 over expression persists for at least one year after HAART is started (178).

Effective antiretroviral therapy is also associated with an increased proliferative response to non-HIV specific antigens and mitogens (156,170-174,179). In contrast, proliferative response to HIV-specific antigens is rarely restored with effective antiretroviral therapy (180). Little data exists pertaining to HCV-specific antigen proliferation following the introduction of HAART. Valdez *et al* did not demonstrate a difference in proliferative response to HCV NS3 or core protein between untreated HIV-HCV co-infected subjects

and those on antiretroviral therapy however the sample size was small, HIV RNA virologic suppression incomplete, and duration of antiretroviral therapy undefined (101). The absolute number of CD4 T lymphocytes was found to correlate with lymphocyte proliferation to NS3 stimulation, which suggests that with HAART-induced immune restoration, HCV-specific cell-mediated immunity may be improved. Despite antiretroviral therapy for at least six months, overall CD4 T lymphocyte IFN-γ production in response to HCV antigen (i.e. core , NS3, NS4) stimulation was poor in HIV-HCV co-infected subjects (102). If HCV-specific immunity is improved with HAART. then a long period of virologically potent therapy may be required.

2.0 Methods

2.1 Study Population

This was a retrospective study of HIV-HCV co-infected individuals followed at The Ottawa Hospital Immunodeficiency Clinic and Ottawa Hospital (General Campus) Viral Hepatitis Clinic. Subjects were identified by chart review. Charts were evaluated from January 1996, which corresponds with the time in which Highly Active Antiretroviral Therapy (HAART) came into use, until August 2001 when patient recruitment for this investigation ceased. HAART was defined as treatment with at least three antiretrovirals of which at least one was a protease inhibitor or non-nucleoside reverse transcriptase inhibitor (N.B. subjects on two nucleoside reverse transcriptase inhibitors with HIV RNA levels suppressed below 500 copies/ml were also included in this cohort). Medical records were reviewed to determine HCV serostatus. Of individuals identified as HIV infected and HCV positive, those subjects who received HAART, remained on therapy for a minimum of six months, and achieved maximal virologic suppression at least once in the first year of therapy (defined as HIV RNA <500 copies/ml) were selected. Subjects were excluded from analysis if baseline or month six quantitative HIV RNA results were not available. Subjects were excluded from analysis if they received immune suppressive medications or interferon-based therapy for HCV infection during the period of evaluation.

2.2 Data Collection and Database Creation

The charts of subjects meeting enrolment criteria were reviewed and the appropriate data extracted. Information pertaining to patient demographics (i.e. age, sex), risk factors for HIV and HCV infection, co-infection with hepatitis B virus, and alcohol consumption was gathered. Date of HAART initiation, antiretroviral medications used, and prior antiretroviral medication use were gathered. HIV RNA level, CD4 and CD8 T lymphocyte count, alanine aminotransferase, aspartate aminotransferase, and bilirubin measures at baseline, month 1, 3, 6, 9 and 12 of HAART therapy were collected. CD4 and CD8 T lymphocyte count results were recovered from The Ottawa Hospital-General Campus Hematology Laboratory if missing from chart records. The measures closest to the first day of therapy were entered as the baseline value. The measure closest to months 3, 6, and 12 (range +/- 1 month) was used at each subsequent time point.

2.2.1 Variable Definition

HIV RNA Level (see Table 3)- Values reported by the Chiron system to be greater than the upper limit of detection (>500,000 HIV RNA copies/ml) were recorded as 500,001 HIV RNA copies/ml). Values below the limit of detection (<500 HIV RNA copies/ml- Chiron 2.0; <50 HIV RNA copies/ml- Chiron 3.0 were recorded as 499 and 49 HIV RNA copies /ml, respectively. The concordance of these two generations of quantitative HIV RNA assays is 80% (181). All but three subjects were found to have HIV RNA below 50 copies/ml by the Chiron 3.0 assay. HIV RNA levels were transformed to $\log_{10}$. This is a standard method of expressing this value.

HCV RNA Level- Values reported by the Cobas System to be greater than the upper limit of detection (>8.5 x 10^6 IU/mL) were recorded in the database as 8.5 x 10^6 IU/mL. Values reported by the Cobas System to be below the lower limit of detection (<600 IU/mL) were recorded as 600 IU/mL). Undetectable results were entered as 0 IU/mL. HCV RNA levels were transformed to $\log_{10}$. This is standard practice for analysis and interpretation of quantitative HCV RNA data.

HCV RNA Level Change from Baseline-The HCV RNA level measured prior to initiation of HAART was used as markers to which subsequent measures were compared. The literature suggests that HCV RNA levels are relatively stable over time in the absence of new antiretroviral therapy justifying this approach (see Introduction- Section 2). Changes in HCV RNA level at month 3, 6, and 12 were calculated by subtracting the $\log_{10}$ transformed HCV RNA level at each time point from the $\log_{10}$ transformed HCV RNA level at baseline. This is standard practice when reporting changes in HCV RNA level.

Alcohol Use- This dichotomous variable referred to alcohol use from the time of initiation of HAART to the last time point evaluated (i.e. month 6 or month 12). The alcohol categories are:

a) <50 grams alcohol per day (i.e. < 3 alcoholic beverages per day)

b) ≥50 grams alcohol per day (≥ 3 alcoholic beverages per day)

This is the standard cut point used by those investigating the relationship between HCV and alcohol. The maximum amount of alcohol per day consumed during the period of evaluation was used to determine classification.

In order to quantitate alcohol use, a complete review of all clinic chart records was conducted. At the clinic visits, alcohol consumption is quantitated by determining alcohol consumption per day, per week, and per month. The type of alcohol being consumed is also recorded. This information is entered into the chart baseline data form at the initial visit and in the progress notes at each subsequent visit. A standardized questionnaire the quantitate alcohol consumption was not used in the clinic during the period of investigation.

Intravenous Drug Use (IVDU)- This dichotomous variable referred to any prior IVDU before the initiation of HAART.

Chronic Hepatitis B Virus Infection- Defined as hepatitis B surface antigen (HBsAg) positive at the time of initiation of HAART.

Antiretroviral Exposure History- Subjects with any prior exposure to nucleoside reverse transcriptase inhibitors, non- nucleoside reverse transcriptase inhibitors, and/or protease inhibitors were classified as treatment-experienced. Those without prior antiretroviral exposure were defined as treatment naïve.

Table 3

List of Variables

Variable	Variable Type	Source	Details
Age	continuous	chart	-
Sex	dicotomous	chart	-
History of IVDU	dicotomous	chart	No active use at time of initiation of HAART
Chronic Hepatitis B virus infection	dicotomous	chart	HsAg positivity indicative of chronic infection
Alcohol Consumption	dicotomous	chart	$<$ or $\geq$ 50 grams alcohol / day
HIV RNA	continuous	Chart, Virology Laboratory Database	System Used: Chiron 2.0, Chiron 3.0
CD4 T Lymphocyte Count	continuous	Chart, Hematology Laboratory	-
CD8 T lymphocyte Count	continuous	Chart, Hematology Laboratory	-
Antiretroviral Naive	dicotomous	chart	-
Protease Inhibitor Containing Regimen	dicotomous	chart	-
Non-Nucleoside Reverse Transcriptase Inhibitor Containing Regimen	dicotomous	chart	-
AST level	continuous	chart	-
ALT level	continuous	chart	-
Bilirubin	continuous	chart	-

2.3 Quantitative HCV RNA Data

Two-milliliter plasma aliquots are routinely separated from blood samples obtained for HIV RNA level determination at the CHEO Virology Laboratory and placed in frozen storage (-70°C). These samples were utilized to determine quantitative HCV RNA levels prior to initiation of HAART (baseline), and at 3, 6, and 12 months thereafter. These time points were selected because specimens were available at these intervals (N.B. it is recommended to measure HIV RNA level following introduction of HAART at these intervals). The Cobas Amplicor HCV Monitor™ Test, Version 2.0 (Roche Diagnostic Systems, Inc.) was used to determine quantitative HCV RNA levels. An estimated value was reported as below or above the limits of detection (<600 IU/ml and >850,000 IU/ml. respectively). If no virus was detected, a negative result (hereafter referred to as undetectable HCV RNA level) was reported. Subjects were excluded if baseline or month 6 quantitative HCV RNA specimens could not be located.

2.4 Statistics

2.4.1 Sample Size

An absolute change in HCV RNA of 0.5 $\log_{10}$ at six months is considered biologically significant (see Introduction-Sections 1, 2). It was calculated that a sample size of 50 subjects would have approximately 80% power to detect a 0.5 $\log_{10}$ absolute difference in HCV RNA level between baseline and 6 months (assuming a standard deviation of 1.25 $\log_{10}$ copies/ml) [see Table 1 (Appendix) for a description of the sample size calculation].

2.4.3 Statistical Analysis

Descriptive analysis of the HIV-HCV co-infected study population comprising this database was performed. Mean and median age, CD4 and CD8 T lymphocyte count, $\log_{10}$ HIV RNA and $\log_{10}$ HCV RNA levels, transaminase (ALT, AST) and bilirubin levels were calculated at baseline and at each subsequent time point.

The difference in $\log_{10}$ HCV RNA levels between baseline and each time point was assessed by two-sided paired t-test.

Baseline $\log_{10}$ HCV RNA levels were not normally distributed. Therefore, nonparametric tests (Mann-Whitney, Sign Test) were used to investigate the association between clinically relevant variables and baseline $\log_{10}$ HCV RNA levels.

The differences in $\log_{10}$ HCV RNA levels between baseline and each time point were normally distributed. Therefore, linear regression analysis were use to investigate the

association between clinically relevant variables and change in HCV RNA level from baseline at months 3, 6, and 12. As planned *a priori*, the variables assessed included:

- sex
- age at initiation of HAART
- alcohol use
- baseline CD4 T lymphocyte count
- baseline CD8 T lymphocyte count
- baseline HIV RNA level
- protease inhibitor(s) inclusion in HAART regimen
- non-nucleoside reverse transcriptase inhibitor inclusion in HAART regimen
- antiretroviral exposure history (naïve vs. experienced)

Post hoc evaluation of CD4 T lymphocyte count at baseline and the change in this parameter following the initiation of HAART was conducted by ANOVA and linear regression analysis.

Analysis was conducted using SPSS Version 11.0.

2.5 Ethics

This protocol was approved by The Ottawa Hospital Research Ethics Board prior to utilization of the specimens stored at the Provincial Virology Laboratory (CHEO). The Provincial Virology Laboratory (CHEO) is a reference laboratory for The Ottawa Hospital. It is within the jurisdiction of The Ottawa Hospital Research Ethics Board to review studies in which laboratory work in conducted at this laboratory. Patient confidentiality was maintained throughout the course of this study.

The key ethical issues pertaining to this study include patient confidentiality and the use of frozen blood specimens for research. Patient confidentiality was protected by assigning subjects a code that was used during data analysis (as a opposed to using patient names). All data is stored securely in a locked room.

The use of frozen blood specimens drawn for one purpose (in this case, HIV RNA quantitation) for a different purpose has raised ethical questions regarding the need for patient consent. It was not possible to contact all subjects for permission prior to utilizing their blood specimens for HCV RNA testing. Following discussion with The Ottawa Hospital Research Ethics Board and the Provincial Laboratory based at the CHEO, it was decided that the physicians originally requesting these blood specimens to be drawn would provide written consent on their patients' behalf, for the testing of these specimens. Testing of these specimens for purposes other than HCV RNA quantitation did not occur. Results were reported by study patient code to protect confidentiality.

3.0 Results

3.1 Study Population

The Ottawa Hospital Immunodeficiency Clinic has provided care to HIV-infected patients living in the Eastern Ontario and Western Quebec regions for a decade. Subjects are in general referred to this multidisciplinary clinic by their family physicians. Approximately 1200 patients currently have active charts at this clinic. This represents the vast majority of HIV-HCV co-infected subjects in Ottawa and the surrounding region. For this reason, this study cohort is representative of the HIV-HCV co-infected population receiving medical care in the Ottawa area.

Between June 1999 and August 2001, all available charts located at The Ottawa Hospital Immunodeficiency Clinic were reviewed to determine HCV serostatus. Of 1200 patient names entered on The Ottawa Hospital Immunodeficiency Clinic roster, 973 charts were located (Figure 1). The remaining charts were in off site storage either because the subject had died or had not returned for a clinic visit in the preceding two years. Retrieval of these charts was not attempted given that the era in which they were followed in clinic (pre-1997) predated routine HIV RNA quantitation (i.e. no frozen specimens in which to measure HCV RNA level were available for these subjects). It also predated the routine use of HAART. The characteristics of these subjects is uncertain aside from the likelihood that they were not treated with HAART for at least 6 months, as was the cohort eventually selected for this study (N.B. the widespread adoption of HAART began in mid-1996).

Of the 973 charts located, documentation of HCV testing was present in 862 (89%). Two hundred and thirty-six subjects were identified as being HCV seropositive. Of these 236 subjects, 108 received HAART while followed at The Ottawa Hospital Immunodeficiency Clinic.

Of these 108 subjects, 65 individuals received HAART for a minimum of 6 months and achieved HIV RNA suppression below 500 copies/ml at least once during this period of time (Table 4). These charts were completely evaluated for demographic information, laboratory measures, and antiretroviral therapy history.

Of the remaining 65 individuals, 50 were included in the final database. Frozen plasma specimens could not be located in 9 of 65. Six of 65 lacked specimens for month 6 measurement of HCV RNA. A comparison of baseline characteristics failed to identify significant differences between those 50 included in the final cohort and those 15 who were excluded (Table 5).

Figure 1

Flow Chart Describing Final Study Cohort

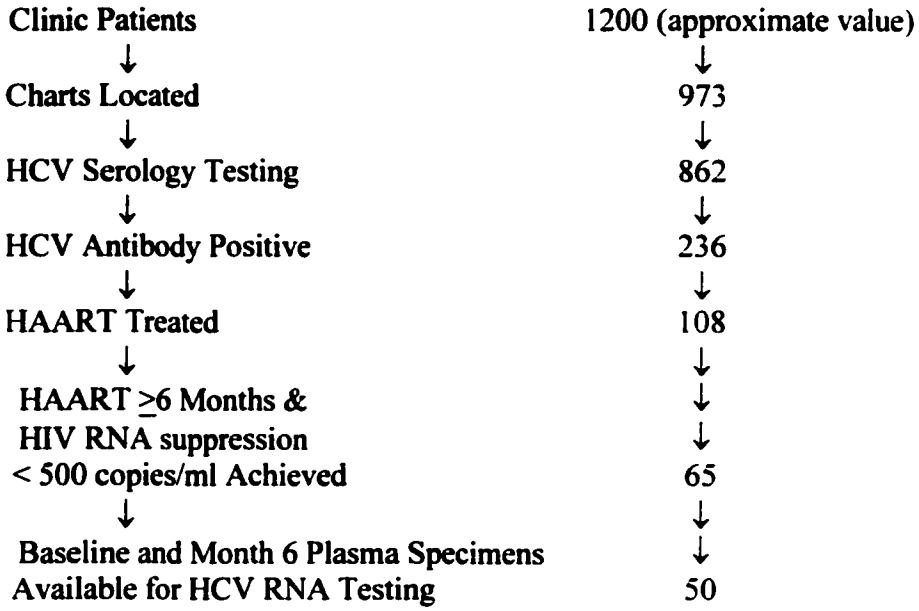


Table 4

Reason for Exclusion

Reason for Exclusion	N
No Baseline HIV RNA level	22
HAART Duration < 6 Months	9
Failure to Suppress HIV RNA <500 copies/ml	10
Insufficient Chart Data	2
Total Excluded	43 / 108

Table 5

Baseline Cohort Characteristics of Included and Excluded Subjects

Parameter	Cohort (n=50)	Excluded (n=15)
Age (Mean, Median)	38, 37	34, 35
Sex (Male: Female)	39:11	13:2
Mean Baseline CD4 Count	330 (SD* 260)	307 (SD 209)
Median Baseline CD4 Count	259	312
Mean Baseline HIV RNA	4.37 (SD 0.89)	4.23 (SD 0.75)
Median Baseline HIV RNA	4.44	4.29
Protease Inhibitor-Based HAART	39:11	12:3

*SD=standard deviation

3.2 Descriptive Analysis of Study Cohort

Baseline characteristics of this cohort were determined (Tables 6,7). Noteworthy observations include: 1) preserved immune function (mean CD4 count > 200 cells/μL), 2) normal bilirubin levels suggesting that liver function in this cohort is preserved, 3) most subjects acquired HCV and HIV infection by IVDU, 4) most subjects were initiated on PI-based HAART regimens, 5) a high proportion of subjects had undetectable baseline HCV RNA levels, and 6) a high proportion of subjects had undetectable HCV RNA levels at each measure.

Table 6

Continuous Baseline Cohort Characteristics

Parameter	N	Mean	SD	Median	Quartiles
Age	50	38.4	5.82	37	34, 41
Baseline HIV RNA (copies/ml)	50	4.37	0.89	4.42	3.80, 4.88
Baseline HCV RNA (IU/ml)	50	4.85	2.55	5.98	4.87, 6.46
Baseline CD4 Count	50	330	260	259	145, 433
Baseline CD8 Count	39	849	596	672	511, 939
ALT (U/ml)	46	51	45.6	36	22,70
AST (U/ml)	46	42	61.8	32	27,55
Bilirubin (ml/M)	45	11	6.1	10	8,12

Table 7

Baseline Cohort Characteristics

Parameter (n=50)	Total	%
History of IVDU	41	82
≥ 50 grams Alcohol per Day while on HAART	9	18
Hepatitis B Infection (HBsAg +)	5	10
Antiretroviral Naive	21	42
PI-based HAART	36	72
NNRTI-based HAART	9	18
NRTI-based Therapy	2	4
PI and NNRTI-based Therapy	3	6
Baseline HCV RNA Level Undetectable	10	20
All HCV RNA Measures Undetectable	6	12

Evaluation of Subjects with Undetectable HCV RNA Levels

Six of 50 individuals had undetectable HCV RNA levels at each time point measured. This can be interpreted to indicate:

a) Persistent, low level viral replication which is below the lower limit of detection for the HCV assay used.

b) previous infection (by virtue of a HCV antibody seropositivity) with subsequent spontaneous clearance of infection (i.e. not chronically infected).

In this study, the former interpretation was assumed.

For the purposes of subsequent analysis it was important to determine whether these subjects differed from the other 44 subjects with detectable HCV RNA levels. The six subjects with undetectable HCV RNA levels were comparable to those of the rest of the cohort (Table 8). Overall, the two groups where similar and therefore combined for subsequent analysis.

Table 8

Subjects with Undetectable HCV RNA Compared to Remaining Cohort

Parameter	HCV RNA Undetectable (n=6)	HCV RNA Positive (n=44)
Age (Mean, Median)	39, 36	38, 38
Sex (Male: Female)	4:2	35:9
Mean Baseline CD4 Count	383 (SD 353)	323 (SD 249)
Median Baseline CD4 Count	280	259
Mean Baseline HIV RNA	3.86 (SD 0.89)	4.44 (SD 0.88)
Median Baseline HIV RNA	4.12	4.48
Mean ALT	21 (SD 7)	55 (SD 47)
PI-based HAART	5	34

In four cases, HCV RNA was undetectable at baseline but subsequent measure(s) were positive following the initiation of HAART (table 9).

Table 9

Description of Individuals with Varying Detectability of HCV RNA Level

Subject	Baseline	Month 3	Month 6	Month 12
1	-	+	-	NM
2	-	+	+	-
3	-	+	+	NM
4	-	NM	-	+

+ positive HCV RNA level, - undetectable HCV RNA level
NM- not available for measurement

An additional subject was HCV RNA positive at baseline, month 3 and month 6 however at month 12, his HCV RNA was undetectable.

These data suggest that a single undetectable HCV RNA level is insufficient to conclude that chronic HCV infection is not present.

3.3 Evaluation of Predictors of HCV RNA Level at Baseline

3.3.1 Analysis of Key Covariates

The distribution of baseline $\log_{10}$ HCV RNA levels was not normal (data not shown). Therefore, factors potentially influencing baseline $\log_{10}$ HCV RNA levels were evaluated non-parametrically (i.e. Mann-Whitney Test) (Table 10).

Table 10
Dichotomous Covariates as Predictors of Baseline $\log_{10}$ HCV RNA Level

Variable		N	$\log_{10}$ HCV RNA Level			Z	P Value
			Mean	SD	Median		
Sex	Female	11	4.45	2.92	5.64	-0.33	0.74
	Male	39	5.00	2.46	5.98		
Antiretroviral Experience	Naive	21	5.67	1.57	5.97	-0.81	0.42
	Experienced	29	4.26	2.95	6.16		
IVDU History	Yes	41	4.68	2.62	5.94	-1.27	0.21
	No	9	5.62	2.16	6.28		
Alcohol Use	≥50 g/day	9	3.38	3.21	5.63	-1.79	**0.077**
	<50 g/day	41	5.18	2.30	6.16		
Median Age at Initiation of HAART (37 yrs)	Above	28	5.07	2.44	6.14	0.37	0.71
	Below	22	4.58	2.71	5.88		
Baseline ALT	≥40 U/L	22	5.93	1.39	6.23	2.11	**0.035**
	<40 U/L	24	3.88	2.94	5.31		
Hepatitis B Status	Yes	5	4.95	2.80	5.98	-0.20	0.85
	No	45	4.84	2.55	5.97		
Median Baseline HIV (4.42 copies / mL)	Above	25	5.18	2.13	5.94	-0.30	0.76
	Below	25	4.53	2.91	6.16		
CD4 T Lymphocyte Count (Analysis 1) [1]	≤ 200	20	4.52	2.80	6.03	0.61	0.54
	> 200	30	5.08	2.39	5.98		
CD4 T Lymphocyte Count (Analysis 2) [2]	≤ 50	7	4.05	3.08	5.95	-0.49	0.62
	> 50	43	4.98	2.47	5.98		
Median Baseline CD8 T Count (637 cells / μL)	Above	20	5.90	1.49	6.37	1.92	**0.055**
	Below	19	4.10	2.99	5.89		

[1] HIV infected subjects are defined as have AIDS at 200 cells/μL
[2] 50 cells/μL used to demark profound immune compromise

ALT levels greater than the upper limit of normal and alcohol consumption less than 50 grams per day were found to be statistically significant predictors of higher baseline $\log_{10}$ HCV RNA level. A trend toward higher baseline $\log_{10}$ HCV RNA level was found with CD8 T lymphocyte counts above the median level. Sex, age, antiretroviral experience. CD4 T lymphocyte count, and baseline $\log_{10}$ HIV RNA level were not found to predict baseline $\log_{10}$ HCV RNA level. Hepatitis B status and history of IVDU were not found to be significant covariates in predicting $\log_{10}$ HCV RNA levels. These analyses were limited by the fact that few subjects were hepatitis B infected and most acquire HCV infection as a result of IVDU.

The baseline HCV RNA level was lower in those subjects drinking $\geq$ 50 g alcohol per day at the time of initiation of HAART compared to those consuming less than this amount (Table 10). This is contrary to other studies, which suggest that HCV RNA levels are higher in those consuming excess alcohol (52,89-91).

Four of the 9 (44%) subjects with consumption of $\geq$50 grams of alcohol per day while on therapy had undetectable baseline HCV RNA levels. Six of 41 (15%) subjects consuming < 50 grams of alcohol per day had undetectable baseline HCV RNA levels. This approached statistical significance by Fisher's Exact Test (p=0.065). This work suggests that in this cohort, consumers of $\geq$50 grams of alcohol per day were more likely to have "mild" chronic HCV infection. This characteristic is likely responsible for the relationship between alcohol consumption and baseline HCV RNA level observed in this study.

3.4 Evaluation of log_{10} HCV RNA levels following initiation of HAART

3.4.1 Descriptive Analysis of log_{10} HCV RNA Level over 12 Months

The log_{10} HCV RNA level increased between baseline and the third month of HAART and then gradually declined toward baseline over the ensuring months (Tables 11, 12). At three months following the initiation of HAART, the log_{10} HCV RNA level increased by a median 0.29 log_{10} (mean 0.55 log_{10}). The log_{10} HCV RNA level declined at subsequent time points.

Table 11
Evaluation of Median log_{10} HCV RNA Levels Following Initiation of HAART

	N	Median (HCV RNA log_{10})	Quartiles	Median Change from Baseline
HCV RNA Baseline	50	5.98	4.87, 6.46	-----
HCV RNA Month 3	38	6.45	5.84, 6.64	0.29
HCV RNA Month 6	50	6.34	5.68, 6.64	0.23
HCV RNA Month 12	25	6.19	4.74, 6.71	0

Table 12
Evaluation of Mean log_{10} HCV RNA Levels Following Initiation of HAART

Pair	N	Mean (HCV RNA log_{10})	SD	Mean Difference
Baseline to Month 3	38	4.85	2.49	0.55
		5.50	2.25	
Baseline to Month 6	50	4.85	2.55	0.43
		5.28	2.40	
Baseline to Month 12	25	4.68	2.74	0.29
		4.97	2.61	

Because of skewed distribution, Wilcoxon Signed Rank Test was utilized to compare the difference in HCV RNA level between time point (Table 13). Significant differences in HCV RNA level between baseline and month 3 ($p<0.001$) and between baseline and month 6 ($p=0.008$) were observed.

Table 13

Evaluation of $\log_{10}$ HCV RNA Level Between Time Points

Comparaison	N	Z	P-Value*
Baseline to Month 3	38	3.63	[illegible]
Baseline to Month 6	50	2.64	[illegible]
Baseline to Month 12	25	1.20	0.232

*Wilcoxon Signed Ranks Test

3.4.2 Evaluation of the Direction of Change from Baseline and Consistency of that Change in HCV RNA Levels at Months 3 and 6

This evaluation was conducted by using the Sign Test. Following initiation of HAART. there was an increase in HCV RNA level between baseline and month 3 (Table 14). Thereafter, a trend toward a decline from previous HCV RNA levels was observed.

Table 14

Evaluation of Direction of Change in $\log_{10}$ HCV RNA Level Between Time Points

Direction of Change	N	Positive	No change	Negative	Z	P Value
Baseline to Month 3	38	23 (60%)	6 (16%)	9 (24%)	2.30	[illegible]
Baseline to Month 6	50	30 (60%)	9 (18%)	11 (22%)	2.81	[illegible]
Baseline to Month 12	25	11 (44%)	5 (20%)	9 (36%)	-----	0.824*
Month 3 to Month 6	38	11 (29%)	7 (18%)	20 (53%)	-1.44	0.15
Month 6 to Month 12	25	6 (24%)	4 (16%)	15 (60%)	-----	0.078*

* Binomial distribution used

3.4.3 Predictors of HCV RNA Level Change from Baseline

The distribution of the change in $\log_{10}$ HCV RNA from baseline to each of the time points was normal. Therefore predictors of change in $\log_{10}$ HCV RNA level from baseline were evaluated by linear regression (Table 15).

Table 15
Continuous Variables as Predictors of Change in $\log_{10}$ HCV RNA Level from Baseline*

Variable	Month 3			Month 6			Month 12		
	N	Beta	P Value	N	Beta	P Value	N	Beta	P Value
Age	38	-0.008	0.96	50	-0.044	0.76	25	0.089	0.67
CD4 T cell	38	-0.11	0.52	50	-0.24	0.10	25	-0.15	0.47
CD8 T cell	31	-0.13	0.50	39	0.25	0.13	17	0.191	0.46
Baseline HIV RNA	38	0.19	0.26	50	0.34	[illegible]	25	0.410	0.042
Baseline HCV RNA	38	-0.43	[illegible]	50	-0.38	[illegible]	25	-0.47	0.019
ALT	35	-0.22	0.21	46	0.009	0.95	23	-0.10	0.67

*Univariate Linear Regression

Age, baseline CD4 and CD8 T lymphocyte count, and baseline ALT were not predictive of the change in HCV RNA level from baseline. Baseline HIV RNA level was positively correlated with the size of HCV RNA change from baseline at 6 and 12 months. The size of the change in HCV RNA from baseline was inversely correlated with the baseline HCV RNA level.

Two dichotomous variables were found to be significant predictors of change in HCV RNA level from baseline. These included baseline CD4 T lymphocyte count (Table 16) and alcohol consumption (Section 3.4.4).

Table 16
Change in HCV RNA Level as a Function of Baseline CD4 T Lymphocyte Count

Variable	Month	Category	N	Mean (Standard Deviation)	P
CD4 T cell	3	≤50 cells/μL*	6	0.81 (1.51)	0.56
		>50 cells/μL	32	0.51 (1.10)	
	6	≤50 cells/μL	7	1.64 (2.63)	0.011
		>50 cells/μL	43	0.23 (0.99)	
	12	≤50 cells/μL	2	3.27 (5.16)	0.047
		>50 cells/μL	23	0.03 (1.84)	
	3	≤200 cells/μL**	16	0.71 (1.48)	0.48
		>200 cells/μL	22	0.44 (0.86)	
	6	≤200 cells/μL	20	0.77 (1.64)	0.15
		>200 cells/μL	30	0.20 (1.16)	
	12	≤200 cells/μL	8	1.02 (2.41)	0.27
		>200 cells/μL	17	-0.054 (2.15)	

* < 50 CD4 cells/ml is indicative of profound immune compromise
** < 200 CD4 cells/ml is a criteria for diagnosis of AIDS

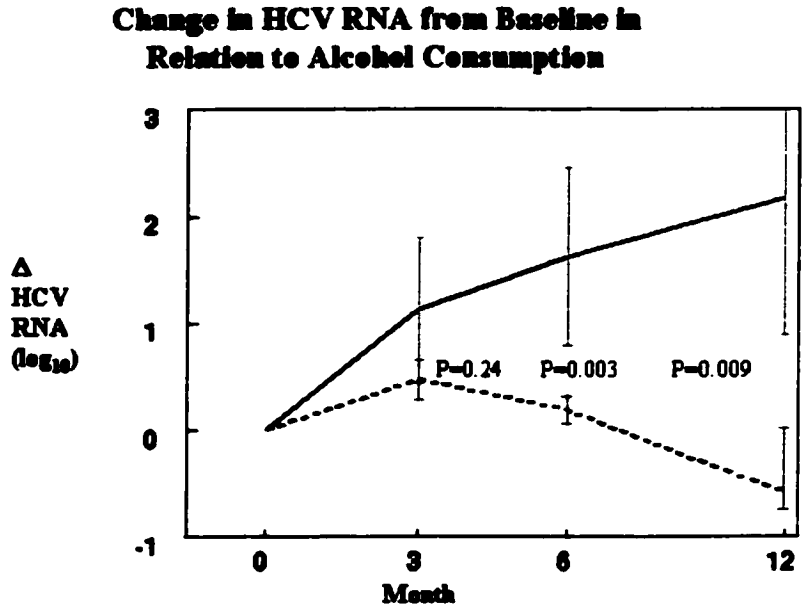
Several other dichotomous variables were evaluated as predictors of change in HCV RNA level from baseline by ANOVA including:

- Sex
- Protease Inhibitor inclusion in HAART regimen
- Baseline ALT level (normal versus elevated)

These variables were not found to predict or be associated with change in HCV RNA level (Appendix B).

3.4.4 Evaluation of the Relation of HCV RNA Change from Baseline and Alcohol Consumption

Figure 2



——— ≥50 grams alcohol per day
-------- <50 grams alcohol per day

Table 17

Change in HCV RNA Level as a Function of Alcohol Consumption

Variable	Month	Category	N	Mean	SD	P
Alcohol	3	≥ 50 g /day	5	1.13	1.51	0.24
		< 50 g /day	33	0.47	1.09	
	6	≥ 50 g /day	9	1.62	2.50	0.003
		< 50 g /day	41	0.17	0.85	
	12	≥ 50 g /day	6	2.29	2.98	0.009
		< 50 g /day	19	-0.34	1.58	

At each time point the mean and median change in HCV RNA level from baseline was greater in those consuming > 50 grams of alcohol per day (Figure 2, Table 17).

The change in HCV RNA level from baseline was evaluated in subjects not using excess alcohol (Figure 2, Table 18). A statistically significant mean increase in HCV RNA level at three months following the initiation of HAART was noted. Mean HCV RNA level fell below baseline following twelve months of HAART.

Table 18

Evaluation of Change in HCV RNA Level from Baseline in Subjects not Using Excess Alcohol

Pair	N	Mean	SD	Mean Difference	SD	95%CI	P Value (2-sided)
Baseline to Month 3	33	5.13	2.33	0.47	1.10	0.08, 0.85	0.019
		5.60	2.15				
Baseline to Month 6	41	5.18	2.30	0.17	0.85	-0.10, 0.43	0.217
		5.34	2.31				
Baseline to Month 12	19	5.18	2.39	-0.34	1.58	-1.10, 0.42	0.355
		4.84	2.64				

3.4.5 Multivariate Linear Regression Analysis of Covariates

Alcohol consumption and baseline HCV RNA level were statistically significant by univariate analysis and therefore were entered into a multivariate model (Table 19).

Table 19
Multivariate Analysis of Variables Significantly Associated with Change in $\log_{10}$ HCV RNA Level from Baseline by Univariate Analysis: Model 1

Variable	Parameter Estimate	P Value
Alcohol	-0.33	0.016
Baseline $\log_{10}$ HCV RNA	-0.29	0.035

R=0.49 R^2=0.24

HIV RNA level at baseline was added to this model (Table 20).

Table 20
Multivariate Analysis of Variables Significantly Associated with Change in $\log_{10}$ HCV RNA Level from Baseline by Univariate Analysis: Model 2

Variable	Parameter Estimate	P Value
Alcohol	-0.24	0.072
Baseline $\log_{10}$ HCV RNA	-0.33	0.013
Baseline $\log_{10}$ HIV RNA	0.29	0.027

R=0.57 R^2=0.32

Models in which baseline CD4 T lymphocyte count, CD8 T lymphocyte count, and ALT level were added to the three above covariates did not substantially alter the significance values or increase the R-value.

3.5 CD4 T Lymphocyte Count in HIV-HCV Co-Infection

3.5.1 Evaluation of Key Covariates

Given that several prior investigations suggested poor CD4 T lymphocyte recovery following initiation of HAART, a post hoc evaluation of this was conducted (Figure 2, Table 21).

Figure 3
Mean Change in CD4 T Lymphocyte Count in HIV-HCV Co-Infected Subjects Following the Initiation of HAART

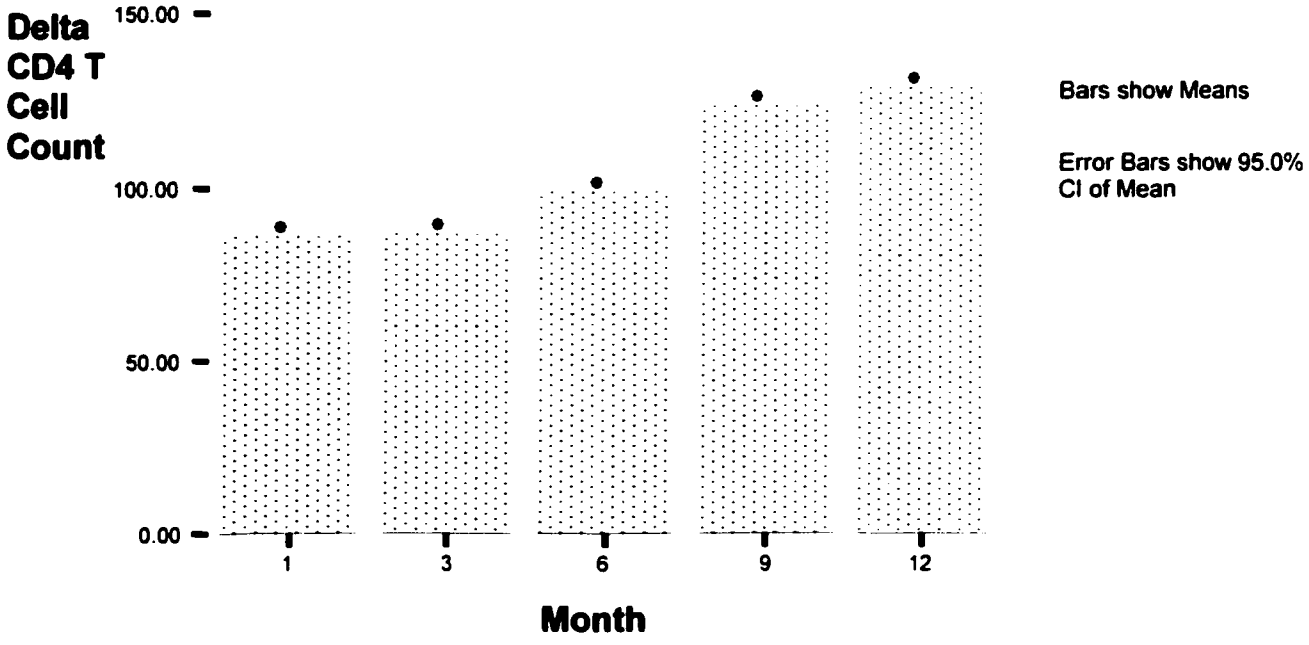


Table 21

Change in CD4 T lymphocyte Count Following Initiation of HAART

Month	N	Mean	SD	Percentile		
				25%	50%	75%
1	30	87	104	13	71	130
3	39	88	86	26	72	133
6	49	100	125	28	94	179
9	22	125	115	48	114	190
12	25	130	119	20	128	219

An increase in CD4 T cell count is observed within the first month of HAART. Thereafter, only a minimal additional increase is observed up to 12 months following the initiation of therapy.

Variables, which may influence the change in CD4 T lymphocyte count from baseline, were evaluated. These included:

Continuous

- Age at initiation of HAART
- Baseline CD4 T lymphocyte count

Dichotomous

- Sex
- Protease Inhibitor use in HAART regimen
- Non-Nucleoside Reverse Transcriptase Inhibitor use in HAART regimen
- HCV PCR status (i.e. positive at least once during period of study versus all measures negative)
- IVDU history
- treatment experienced
- baseline HIV RNA level
- baseline HCV RNA level

No statistically significant relationship was identified between change in CD4 T lymphocyte count from baseline and these covariates. Several of these comparisons were limited by small sample size (Appendix C).

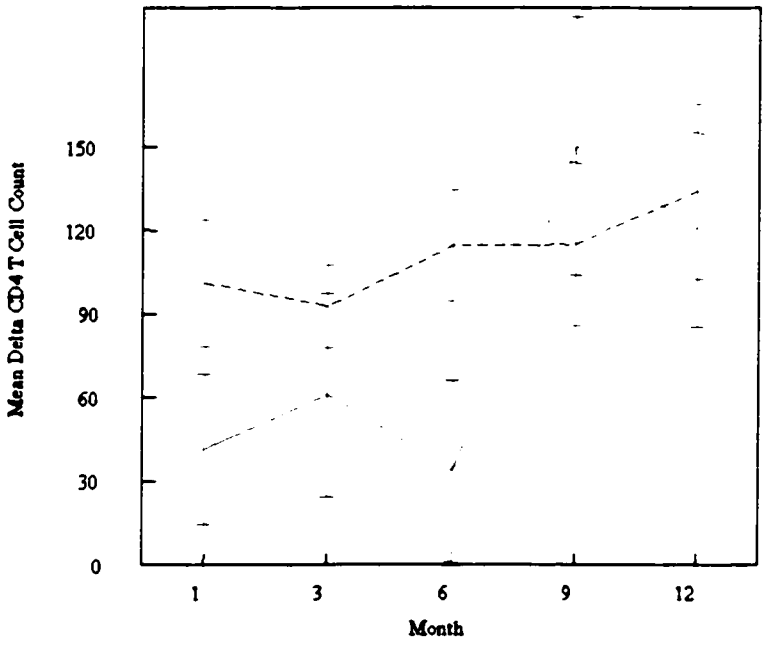
Several trends were identified:

1) At months 1, 3 and 6 a trend toward greater increase in CD4 T cell count in females was noted (Appendix C-Table A-8).
2) No statistically significant difference in CD4 T cell change from baseline was detected between those with HCV PCR positive results and those with HCV PCR below the lower limit of detection at all time points (i.e. generally considered not chronically infected with HCV). A trend at most time points for greater CD4 T cell count increase in the latter group was noted, however (Appendix C-Table A-11).
3) At all points evaluated the mean and median CD4 T cell count increase from baseline was greater in treatment naïve subjects compared to those with prior antiretroviral exposure. This was statistically significant at month 9 (Appendix C-Table A-13).

3.5.2 Change in CD4 T Cell Count as a Function of Alcohol Consumption

Although not statistically significant, a trend toward blunted CD4 T cell increase following the initiation of HAART was observed in those subjects consuming > 50 grams of alcohol per day was noted at months 1, 3 and 6 (Figure 4, Table 22). The effect of this amount of alcohol consumption was less pronounced at months 9 and 12.

Figure 4 Change in CD4 T Lymphocyte Count Following HAART as a Function of Alcohol Consumption*



_____ ≥ 50 grams alcohol per day
-------- < 50 grams alcohol per day

*Standard Deviations included

Table 22

Change in CD4 T Lymphocyte Count in Relation to Alcohol Consumption

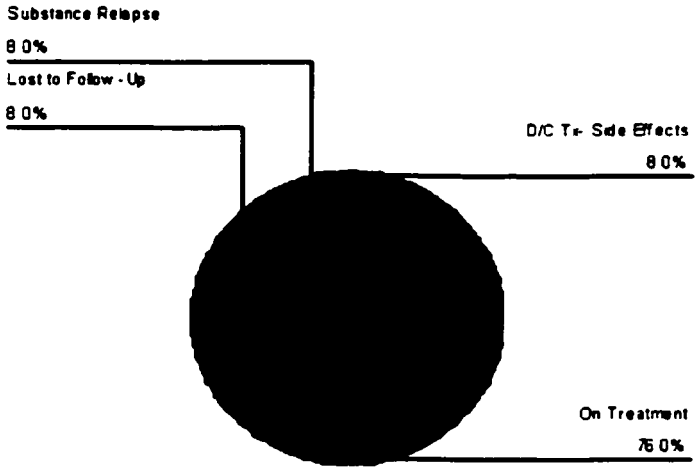
Variable	Month	Category	N	Mean	SD	Median	P Value
Alcohol	1	≥ 50 g /day	7	42	72	10	0.19
		< 50 g /day	23	101	110	74	
	3	≥ 50 g /day	6	61	90	34	0.41
		< 50 g /day	33	93	86	86	
	6	≥ 50 g /day	9	33	98	37	0.80
		< 50 g /day	40	114	127	110	
	9	≥ 50 g /day	6	150	114	180	0.53
		< 50 g /day	16	115	117	100	
	12	≥ 50 g /day	8	120	98	115	0.79
		< 50 g /day	17	134	130	128	

This same analysis was conducted on the 25 subjects followed for the entire 12 months. As in the entire cohort, the CD4 T cell count increase was greater in those consuming < 50 grams of alcohol per day at months 1, 3, and 6 but not statistically significant (Appendix C-Table A-16).

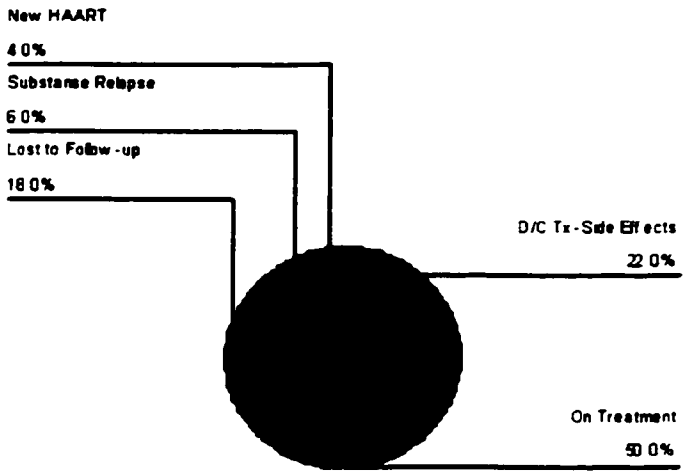
3.6 Patient Outcome

By definition, all subjects included in this cohort were followed for at least six months. The following Pie Charts provide a description of the outcomes following months 6, 12, and 18. As described previously, only half of HIV-HCV co-infected subjects remain on HAART for longer than one year (103).

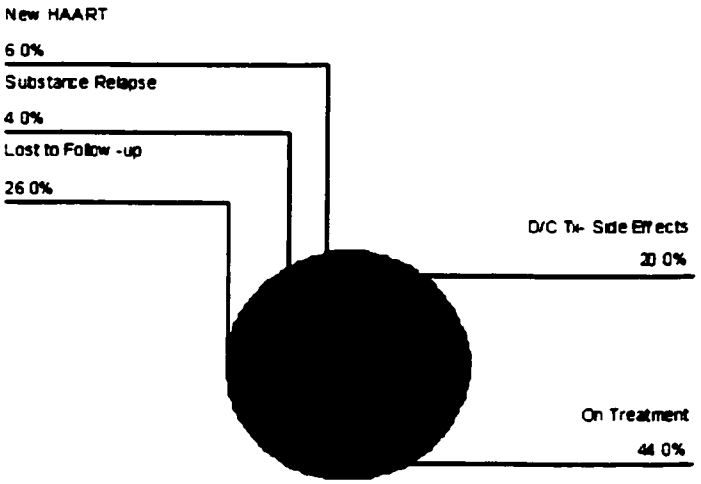
Patient Outcome at Month 6



Patient Outcome at Month 12



Patient Outcome at Month 18



4.0 Discussion

4.1.1 Influence of HAART on HCV RNA Levels over 12 Months

The primary objective of this observational study was to evaluate the effect of HAART on HCV RNA levels in a cohort of HIV-HCV co-infected subjects achieving maximal HIV RNA suppression. Although this has been evaluated in other investigations, these studies have been characterized by small sample size, poorly described populations, incomplete suppression of HIV RNA, and poorly described antiretroviral therapy (32-34,36,37,39,47). In contrast, this study evaluated 50 subjects, all achieving maximal HIV RNA suppression following the initiation of HAART. Because important variables such as sex, age, antiretroviral therapy, alcohol use, and mode of HIV and HCV infection were known, a more detailed evaluation of the effect of HAART on HCV RNA was possible.

As predicted, a 0.55 $\log_{10}$ mean increase in HCV RNA from baseline was observed at month 3 of HAART. Although an initial increase in $\log_{10}$ HCV RNA level has been reported previously (32-34), the increase observed in our cohort was larger. This large increase is likely a result of HAART-related modulation of HCV-specific immunity. I speculate that because HIV RNA suppression was maximal, immune modulation was likewise maximal and that this explains the large initial increase in HCV RNA level relative to other studies. The pathogenesis of this initial increase in HCV RNA level is not entirely clear. Endogeneous levels of interferon, which are elevated in HIV-HCV co-infection subjects and correlate with HCV RNA level (44), are reduced following the initiation of HAART (182-184). Endogeneous interferon likely influences the level of

HCV RNA (77). It has been suggested that HAART-induced reduction in these cytokines may result in a transient increase in HCV RNA (32,34). CD4 T lymphocyte production of interferon-γ plays an important role in control and resolution of HCV infection (185,186). In fact, CD4 T lymphocytes of patients ultimately resolving HCV infection produce more interferon- γ after stimulation with HCV antigen than CD4 T lymphocytes of those who do not (185,186). Improved cytotoxic T lymphocyte (CTL) function with destruction of HCV-infected hepatocytes has been proposed as the mechanism responsible for the initial spike in HCV RNA is some reports (33,47). The absence of a substantial increase in transaminase levels in my cohort, and in other studies (32), suggests against CTL-mediated hepatocyte destruction as the sole mechanism responsible for the transient increase in HCV RNA following initiation of HAART. Direct antiretroviral hepatotoxicity has also been suggested as a mechanism responsible for this initial HCV RNA increase with HAART (41,42). Histological evaluation (33) and the absence of transaminitis in my study, and that of Puoti *et al* (32), indicate that other factors, including altered cytokine milieu, also contribute to this phenomenon. It is questionable whether the amount of HCV RNA released from CTL-mediated destruction of HCV infection hepatocytes or antiretroviral-related hepatotoxicity would be sufficient to account for a greater than 0.5 $\log_{10}$ increase in HCV RNA level lasting several months.

The effect of long term HAART on HCV RNA level is uncertain. There are studies suggesting an increase (33,52-54,57), decrease (34,36,37,51,187), and absence of change (39) in HCV RNA level from baseline. This variability in findings is a result of small sample sizes, insufficient HIV RNA suppression, and the heterogeneity of study cohorts.

The findings of this study are based on a relatively large, well-described cohort with maximally suppressed HIV RNA levels. At six months, the mean HCV RNA level remained elevated (i.e. 0.43 $\log_{10}$ over baseline) but did decline from the peak level reached a month 3. Further reduction in HCV RNA level to near baseline HCV RNA level observed at 12 months. These results are in agreement with those of another recently published evaluation of HIV-HCV co-infected subjects achieving "well-controlled viral replication" (NB- HIV RNA levels in the subjects evaluated in this sub-analysis were not described) (187). In this study 14 of 56 (25%) of subjects were found to have a HCV RNA level below the lower limit of detection (approximately 600 IU/mL) at 12 months following the initiation of HAART.

A possible explanation for the changes in HCV RNA level observed in this study over the initial twelve months of HAART is that endogenous interferon- γ levels, which are high in HIV-HCV co-infected subjects not on HAART (77), fall rapidly following the initiation of HAART therapy which theoretically could result in an immediate increase in HCV RNA level. Restoration of HCV-specific CTL, similar to that of HIV-specific immunity (170-174,179), likely takes much longer to occur. Falling HCV RNA at 6 and 12 months may be indicative of improved HCV-specific CTL.

The primary hypothesis of this study was that HCV RNA level would fall by 0.5 $\log_{10}$ at six months. Although this was not achieved, the negative slope in HCV RNA level from month 3 onward observed in my study is consistent with this hypothesis. This work suggests that with continued maximal HIV RNA suppression, HCV RNA levels continue

to decline. It would appear that a period greater than 6 months is required to restore HCV-specific cell-mediated immunity.

4.1.2 Predictors of Direction and Size of HCV RNA Change

Epidemiological and Statistical Issues

Baseline HCV RNA level and change in HCV RNA level were evaluated to determine if a correlation existed between these measures and several key parameters. These parameters were selected *pre hoc* and were not data driven, as can be the case with *post hoc* analysis (188). Therefore, the identified correlations are less likely to be due to bias. Nonetheless, selection bias is an important consideration when evaluating the applicability of these results to other HIV-HCV co-infected populations. Only 108 (46%) of 236 HIV-HCV co-infected patients followed at our clinic initiated therapy during the period of evaluation. As described previously (103), a large proportion of The Ottawa Hospital Immunodeficiency Clinic HIV-HCV co-infected population were unable to remain on antiretroviral therapy for a prolonged period of time. This is further demonstrated by the observation that only 65 (60%) of 108 subjects initiating HAART remained on therapy for a minimum of six months. It is likely that the burden of co-morbid illness and addiction, common to this population in general, was less in the cohort of 50 evaluated in this study. As described in the Results section (Table 5), subjects meeting the key inclusion criteria (i.e. HIV RNA suppression, duration of HAART-therapy) but not included in the final cohort (n=15) where similar those included in this study (n=50). I am confident that these results are applicable to all long-term, HAART-

treated subjects achieving maximal HIV RNA suppression at The Ottawa Hospital Immunodeficiency Clinic. The final study cohort was selected for similar characteristics.

Follow-up bias, a type of information bias, would not effect comparisons between baseline and month 6 as all cohort subjects had HCV RNA measurements at this time point. In contrast, averaged results of the 38 and 25 subjects with HCV RNA measurements at month 3 and 12, respectively, could potentially differ from those of the 50 subjects measured at baseline and month 6. This was not found to be the case when averaged results of the 38 and 25 subjects at 6 months were compared to the averaged results of the entire cohort at 6 months (data not shown).

Baseline $\log_{10}$ HCV RNA levels were not normally distributed. The data was biphasic: 10 subjects with a baseline $\log_{10}$ HCV RNA level of <600 IU/mL. 40 subjects with a bell-shaped $\log_{10}$ HCV RNA distribution. I assumed that all subjects including those with undetectable $\log_{10}$ HCV RNA levels, were chronically infected with HCV. Based on this assumption, I felt that it was appropriate to analyze all of these subjects together. Non-parameter measures were used to evaluate factors potentially influencing baseline HCV RNA. Parameter measures were used to assess the change in log10 HCV-RNA levels from baseline to months 3, 6, and 12 because these measures approximated normal distribution.

Correlation does not necessarily imply causation. In instances in which a correlation was identified, the causal relationship between the HCV RNA measure and parameter evaluated was considered. Bias and confounding were considered (see below for specific

instances where these factors may have been responsible for an observed correlation). As suggested by Hill, strength, consistency, specificity, temporality, biologic gradient, plausibility, coherence, experimental evidence, and analogy were considered (104).

Alcohol

Alcohol appears to have a substantial influence on immunologic, virologic, and pathologic characteristics of chronic HCV infection. In mice, HCV-specific T-helper and cytotoxic T lymphocytes response as well as cytokine expression is blunted by alcohol consumption (189,190). Furthermore, decreased interferon-γ levels, resulting from alcohol-induced dendritic cell dysfunction (190,191) likely influence HCV RNA levels. Most studies suggest that excess alcohol consumption increases HCV RNA levels (52,90,91) and that this relationship is linear (89). Although analysis of this cohort demonstrated lower baseline HCV RNA levels in those consuming excess alcohol this is likely explained by selection bias (i.e. HIV-HCV co-infected subjects consuming excess alcohol would be less likely to remain on HAART for a over 6 months unless their HCV infection was relatively mild, as suggested by lower baseline HCV RNA level). Analysis indicated that consumers of excess alcohol were more likely to have undetectable HCV RNA levels at baseline (i.e. informational bias) which supports this suspicion (see Results- Section 3.2).

Consumption of greater than 50 grams of alcohol per day was found to be a significant predictor of a large initial $\log_{10}$ HCV RNA spike (1.13 $\log_{10}$ mean increase in HCV RNA level). In comparison, a mean increase of 0.47 $\log_{10}$ HCV RNA was noted in those not

drinking alcohol excessively. This elevation from baseline was sustained at 6 and 12 months in those consuming greater than 50 grams of alcohol per day. This is likely a result of impaired restoration of HCV-specific cellular immune function. In contrast, the HCV RNA level returned to near normal at six months and fell below baseline at twelve months in those not using excess alcohol. No confounders are apparent. There is strength and consistency of these observations. The observations are biologically plausible and coherent. A well-described gradient could not be produced from this particular data set.

I was not able to identify other studies that evaluated the effect of HAART on HCV RNA level change from baseline as a function of alcohol consumption. The immune suppressing qualities of alcohol likely blunt immune restoration following initiation of HAART. If the pathophysiologic explanation for the initial HCV RNA spike is CTL-mediated hepatonecrosis with HCV RNA release, then one would predict that the size of the initial increase in HCV RNA would be decreased because immune restoration would be blunted by alcohol. Given that the size of the initial HCV RNA spike was actually greater in those consuming excess alcohol, this explanation is unlikely. Furthermore, the increase in transaminase level in excess alcohol consumers was modest and similar to those drinking less than 50 grams per day suggesting against direct alcohol-induced hepatotoxicity.

Irrespective of the pathophysiology, the results of this study suggest that alcohol consumption during HAART results in an increase in HCV RNA level. This is likely

indicative of blunted HCV-specific immune restoration. This study provides further evidence of the detrimental effects of alcohol on the nature course of HCV infection (62,89,192). Alcohol consumption must be reduced, if not eliminated, in HCV-infected individuals.

CD4 and CD8 T Lymphocyte Counts

Some (74,80,82,101), but not all studies (193), suggest that baseline $\log_{10}$ HCV RNA level correlates with baseline CD4 T cell count. In those studies reporting a correlation, the association is weak.. In this analysis a correlation between baseline CD4 T cell count and baseline HCV RNA level was not identified. Although the results of this investigation are contrary to the hypothesis that declining immunity results in increased HCV RNA level, they are consistent with other studies evaluating this relationship.

Baseline CD4 T cell count did not influence the size or direction of $\log_{10}$ HCV RNA level change from baseline. This was somewhat surprising as one would predict that the degree of cellular immune competence, as indicated by CD4 T lymphocyte count, would be inversely correlated with the size of the HCV RNA spike. The findings of this study are likely indicative of the fact that HCV-specific, CTL-mediated immune restoration occurs over months to years. It would not be apparent in the first several months following initiation of HAART. Furthermore, CD4 T cell count is but one of a many immune parameters which are altered following initiation of HAART. Measures of HCV-specific cellular immunity including interferon-γ production by CD4 Th1 cells in response to HCV-specific antigen have been found to be more valuable in evaluating the restoration

of HCV-specific immunity in HCV-HIV co-infected subjects following the initiation of HAART (102). In fact, an inverse correlation between HCV core protein specific CD4 Th1 cell frequency and plasma HCV RNA level was identified (r, -0.34; p=0.02). Measurement of endogeneous interferon reduction and correlation with change in HCV RNA level from baseline following the initiation of HAART would be a valuable immune marker to assess.

Baseline $\log_{10}$ HCV RNA level was correlated with baseline CD8 T cell count. No significant relationship between baseline CD8 T cell count and subsequent change in $\log_{10}$ HCV RNA level at months 3, 6, or 12 was observed. A trend toward a negative correlation was noted at each of these time points. No correlation between change in CD8 T cell count from baseline and change in $\log_{10}$ HCV RNA level was identified. At first look the baseline and post-initiation of HAART analysis appear to contradict each other. and the hypothesis that superior immune status correlates with lower HCV RNA level. Knowledge of the function of CD8 receptor expressing cells and the limitations in monitoring CD8 T cell receptor expression help to explain this apparent inconsistency. CD8 is a cellular receptor found on cytotoxic T lymphocytes. Within this group of lymphocytes, there are many subsets with differing function, demarked by other specific cellular receptors. For this reason, monitoring total CD8 T lymphocyte count is of some use in determining overall cellular immune function but is not of great value in determining immune status to specific types of organisms or individual microbes. Furthermore, the qualitative function of a CD8 T lymphocyte is impaired in HIV-infected subjects not on HAART and is improved once therapy is initiated (148,149). For this

reason, similar absolute CD8 T lymphocyte counts in these two populations is not necessarily indicative of similar quality of immune function. Greater understanding of HCV-specific CD8 T lymphocyte activity in HIV-HCV co-infected subjects and modulation of this by HAART, vaccines, and/or immune stimulating molecules may have a significant impact on the ability to successfully eliminate HCV infection.

Baseline HIV RNA level

Baseline HIV RNA level was found to correlate with the size of $\log_{10}$ HCV RNA change from baseline at month 6 and month 12. This remained statistically significant when baseline $\log_{10}$ HCV RNA level and alcohol consumption were included in a multivariate regression model. It is likely that high baseline HIV RNA level is a surrogate marker for impaired HCV-specific immunity. Therefore, these results are likely an example of confounding. Both HCV RNA and HIV RNA levels are dependent on the degree and breadth of cell-mediated immune function. I speculate that the observed larger and longer persisting elevation in HCV RNA level from baseline in individuals with higher baseline HIV RNA is a result of greater impairment in immune function. These subjects would be resistant to the immune restorative effects of HAART. With this delayed improvement in HCV-specific CTL, HCV RNA levels would remain elevated over baseline for a longer period of time.

Baseline HCV RNA Level

The baseline $\log_{10}$ HCV RNA level was inversely correlated with the size of change in $\log_{10}$ HCV RNA level from baseline at month 3, 6 and 12. This remained significant by

multivariate analysis controlling for alcohol consumption and HIV RNA level at baseline. Although the upper limit of detection of the quantitative assay utilized in this analysis was 8,500,000 IU/ml, few measures exceeded this limit; therefore these results do not represent a "ceiling effect". As endogenous interferon-γ levels may inversely correlate with HCV RNA levels, measurement of this cytokine prior to HAART and following initiation of therapy may help to explain this observation. I predict that individuals with low baseline HCV RNA levels have relatively high endogenous interferon-γ levels. Following initiation of HAART, the relative reduction in endogenous interferon-γ may be greatest in those with high pre-treatment levels (i.e. low baseline HCV RNA level). The change in HCV RNA level from baseline would be most pronounced in these individuals.

Sex

Immune function differs in males and females. For example, female sex is a well-known risk factor for several immune-mediated diseases including rheumatoid arthritis, systemic lupus erythematosis, and multiple sclerosis (194). Female sex is a predictor of slower progression of HCV-induced liver fibrosis and of improved response to interferon and ribavirin antiviral therapy. In this study, sex did predict baseline $\log_{10}$ HCV RNA level or the characteristics of $\log_{10}$ HCV RNA change. Investigation of more specific markers of HCV immunity in HIV-HCV co-infected subjects following initiation of HAART may reveal differences in immune restoration between the sexes not identified by the relatively crude measure of HCV RNA level. Also, a larger sample size would reduce the likelihood of Type II error.

Age

A decline in many aspects of immune function occurs with aging (195,196). This is a primary reason why infections and malignancies are more frequent in the elderly. In the treatment of HCV, the likelihood of achieving a sustained virologic response diminishes with increasing age (59). Furthermore, increasing age is associated with declining immune function and reduced immune recovery following initiation of HAART (187). For these reasons, age was evaluated as a variable influencing HCV RNA level. Age was not found to correlate with baseline HCV RNA level. The range of age was relatively narrow which may explain this negative finding.

Antiretroviral Class

No direct anti-HCV activity has been identified with nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitor, and protease inhibitors. HIV antiretroviral medications themselves could potentially influence the direction and degree of change in HCV RNA level following the initiation of HAART. Puoti *et al* suggested no difference in the direction or degree of HCV RNA change between protease inhibitor and non-nucleoside reverse transcriptase inhibitor-based therapy (32). This may be an example of a type II error considering the small sample size. In my study, the classes and types of antiretrovirals used for HIV therapy were not identified as predictors of change in $\log_{10}$ HCV RNA levels. Specifically the inclusion of protease inhibitors, for which there is some evidence of superior immune restorative properties in comparison to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase

inhibitors (48,197), was not identified as a factor predicting the size or direction of HCV RNA change from baseline. Although treatment bias may influence these results (i.e. current practice in some clinics is to avoid protease inhibitors in HCV-infected subjects with evidence of active disease for fears of hepatotoxicity), this is unlikely as protease inhibitors avoidance was not standard of practice during the study evaluation period.

4.1.3 Relevance

The results of this study support the practice of deferring HCV antiviral therapy until after HAART has been initiated. HCV RNA level predicts the likelihood of sustained response to interferon and ribavirin antiviral therapy (defined as plasma HCV RNA negativity six months following completion of antiviral treatment) (58-61). In subjects with HCV RNA levels greater than 2.0×10^6 HCV RNA copies/ml by National Genetics Institute HCV SuperQuant system (equivalent to about 2.0×10^5 HCV RNA copies/ml by the Roche AMPLICOR HCV Monitor system), the sustained response following 48 weeks of interferon and ribavirin was 36% compared to 43% for those with HCV RNA levels below this level (59). It is reasonable to assume that the same is true in HIV-HCV co-infected subjects. If HCV RNA level is reduced following the initiation of potent and durable antiretroviral treatment, then the likelihood of responding to interferon and ribavirin therapy with sustained clearance of HCV RNA may be increased.

HCV RNA is higher in HIV infected subjects (16,74,77). This is likely a result of impaired HCV-specific immune. This impairment may explain why poorer sustained virologic response rates are seen in HIV-HCV co-infected subjects than in HCV

monoinfected subjects (198,199). Although not well studied, it is reasonable to predict that individuals with a relatively functional immune system, either because of early HIV infection or because of the immune restoring effects of HAART therapy, would be better able to respond to HCV antiviral therapy with a sustained virologic response. The findings of this study, in combination with those of others (101,102) suggest that HCV-specific immunity is improved with HAART. Benhamou *et al* identified significantly less liver fibrosis, reduced inflammation and a reduction in hepatitis fibrosis rate in HIV-HCV co-infected subjects receiving HAART in comparison to those who were not (62). These results are likely a result of improved immune control of HCV infection as a result of HAART-induced HIV virologic suppression. The results of this thesis and the above described literature support the practice of first treating HIV infection , and then, once HCV-specific immunity has been restored to more normal function, initiating HCV therapy. This approach is predicted to slow HCV induced liver fibrosis and improve HCV sustained virologic response rates.

4.2 Interpretation of Undetectable HCV RNA Results in HIV-HCV Co-Infection

HIV seronegative individuals with positive HCV antibody titres, normal transaminase levels and undetectable HCV RNA levels are not considered to be chronically infected with HCV (200). The results of this evaluation suggest that this assumption may not be correct in HIV-infected subjects. In this cohort of fifty subjects, ten were initially found to be HCV RNA negative by quantitative assay (lower limit of detection approximately 600 IU/ml). Baseline transaminase measures were within normal limits in eight of nine subjects with available results. Four of these ten individuals were found to have detectable plasma HCV RNA with subsequent repeat testing. Of note, three of four had normal transaminase levels at baseline. This, in combination with a negative plasma HCV RNA test, is considered by many to be sufficient evident of previous HCV infection clearance to preclude additional investigation. Review of medical records does not suggest acute re-infection during the period of evaluation. It appears likely that these patients had persistent HCV infection without detectable plasma HCV RNA or elevation in transaminase level at baseline. This status was perturbed by the introduction of HAART which results in an increase in plasma HCV RNA level. Chronic HCV infection is a well-established risk factor for biochemical and clinical by evident HAART-related hepatotoxicity. Clearly, if chronic HCV infection is present then it is important to identify. In order to avoid this antiretroviral-related adverse event, confirmatory repeat plasma HCV RNA testing in HIV-HCV co-infected subjects with an initial negative test, even if associated with normal transaminase levels, is recommended. Furthermore, close

monitoring of hepatic status in all HCV seropostive subjects initiating antiretroviral therapy (including those without detectable plasma HCV RNA) is warranted.

4.3.1 Immunologic Restoration following HAART in HIV-HCV Co-Infected Subjects

Several retrospective investigations have suggested that the CD4 T cell count recovery following initiation of HAART in HIV-HCV co-infected subjects is blunted in comparison to those without HCV infection (187,201). In a prospective evaluation of 1157 subjects (37.2% HCV coinfected), CD4 T lymphocyte count increase at 6, 12, and 18 months was less in the HIV-HCV co-infected population in comparison to HIV infected subjects (187). These populations were well matched for duration of HIV infection, age, sex, and baseline CD4 T lymphocyte count. Unfortunately, this and other studies are limited in that potential confounders of immune restoration are poorly described and not controlled for during analysis. These include alcohol consumption, concurrent substance abuse, nutritional status, adherence to therapy, extent of prior antiretroviral exposure and degree of virologic suppression. In this study of HIV-HCV co-infected subjects with maximal virologic suppression, CD4 T cell recovery was found to be blunted in comparison to most other studies of HCV negative, HIV-infected subjects initiating HAART (48-50,202). Further investigation of this phenomenon was possible because information on the above-described potential confounders of CD4 T cell response to HAART was available.

Alcohol is known to have immune suppressing properties, which effect both humoral and cell-mediated immunity (190,203). Alcoholism is a problem of high prevalence in HCV-infected subjects. It is possible that excess alcohol use is responsible for poor CD4 T cell

recovery following initiation of HAART. Although not statistically significant, the mean increase from baseline in CD4 T lymphocyte count at months 1, 3 and 6 was lower in users of excess alcohol (table 22, figure 4). This finding again demonstrates the negative effect of alcohol on immune restoration. Further analysis of the cohort suggests that alcohol is not entirely responsible for poor CD4 T cell recovery. The individuals not drinking excess alcohol were also found to have poor CD4 T cell responses in comparison to studies of HCV-uninfected recipients of HAART. As well, the mean CD4 T cell increase at 12 months was similar for those drinking less than 50 grams of alcohol per day and those consuming more. This analysis suggests that excess alcohol consumption does not fully account for the blunted CD4 T lymphocyte count response observed in this cohort.

A potential confounder of CD4 T cell response with HAARTmay be concurrent intravenous drug use. This is not well addressed in other studies. Although intravenous drug use was the primary risk factor for HIV and HCV infection in this cohort, none of these subjects were actively injecting at the time of initiation of HAART. Few subjects resumed intravenous drug use immediately prior to discontinuation of treatment (see Results Section 3.6). The results of this analysis can be interpreted without considering the potential confounding influence of this variable. It is possible that prior intravenous drug use is a marker of some other variable, such as infection with another unknown chronic infection, which may influence HAART-induced immune restoration. Hepatitis G virus, which is also transmitted by blood-to-blood exposures, has been suggested to influence the rate of progression of HIV infection (204). Although limited by sample

size, my analysis suggests that acquisition of HCV infection by prior intravenous drug use, in comparison to other modes of infection, does not influence the size of CD4 T cell increase following initiation of HAART in HIV-HCV co-infected subjects.

Although not statistically significant, the CD4 T lymphocyte increase in treatment-experience subjects at each time point was smaller than that of antiretroviral naïve subjects (table A-13). Infection with antiretroviral resistant strains of HIV may partially explain this finding. Lead time bias may also be an important factor. The permissiveness of the immune system to HAART-induced restoration falls as HIV disease progresses (205-207). A group of naïve subjects receiving their first HAART regimen are likely to have less advanced disease than a group of treatment-experienced subjects receiving salvage therapy. Keeping this in mind, CD4 T cell response to HAART was smaller in those treatment naïve individuals evaluated in this study (48-50,202) than in other studies of treatment naïve, HCV-negative antigens (table A-13).

It is possible that HIV-HCV co-infected subjects initiate antiretroviral therapy at a more advanced state of HIV disease. As suggested above, this would increase the likelihood of a poor immune response to HAART. The mean baseline CD4 T cell count in the cohort evaluated in this study was 330 cells/μL. This did not differ by sex or treatment experience status. At this CD4 T cell level, HIV infected subjects are not at high risk for opportunistic infections or other AIDS-defining illnesses. Furthermore, robust immune restoration would be expected in a cohort of HCV negative, HIV-infected subjects

following the initiation of HAART. In this evaluation, poor pre-HAART immunologic status is not responsible for the observed poor CD4 T cell response to therapy.

CD4 T lymphocyte response may also differ by classes of antiretroviral used in HAART therapy (48,197,202). This issue has not been assessed in HIV-HCV co-infection. In this analysis, immune restoration, as measured by CD4 T cell increase from baseline did not differ between protease inhibitor-containing and sparing HAART regimens. Inclusion or exclusion of non-nucleoside reverse transcriptase inhibitors likewise was not found to influence this measure. If antiretroviral class does influence the magnitude of CD4 T cell recovery in HIV-HCV co-infected subjects, the effect is likely small.

Poor adherence to antiretroviral therapy may be responsible for poor CD4 T cell recovery in HIV-HCV co-infected subjects. In this cohort, good adherence can be assumed given that HIV RNA levels were well suppressed over the duration of this study. Although dietary status was not formally evaluated in this cohort, it can be assumed that caloric intake in these subjects was adequate. It is standard of practice in our clinic to ensure stable housing and nutritional status prior to initiating antiretroviral therapy. Other variables, which could theoretically influence immune recovery including sex, age, baseline HCV RNA level, and baseline HIV RNA level, were not found to influence CD4 T cell recovery.

This analysis is limited by the absence of a matched HIV infected and HCV seronegative control arm. The results of this assessment were compared to well-described cohorts

followed at The Ottawa Hospital Immunodeficiency Clinic (202). When compared to this historical cohort, the fifty HAART-treated HIV-HCV co-infected subjects were found to have a blunted CD4 T cell response following the initiation HAART. Another limitation is that the effect of HCV genotype on CD4 cell recovery was not evaluated.

Having accounted for alcohol consumption, adherence to HAART, nutritional status, prior antiretroviral exposure, pre-treatment immune status and use of illicit drugs, I believe that this analysis suggests a direct influence of HCV infection on CD4 T cell restoration following the initiation of therapy. A possible mechanism for this may be persistent immune activation resulting from chronic, uncontrolled HCV infection. Immune activation induced by HCV may partially negate the immune deactivating effects of HAART therapy thereby allowing for continued immune depletion and reduced immune restoration (101). Although HCV infection does not produce a clinically recognized state of immune deficiency, chronic infection may induce subtle deficits in immune function. Antibody concentrations following vaccination for hepatitis A and hepatitis B are lower in HCV infected patients than in HCV seronegative individuals (208,209). This impairment of immune function may also partially explain the blunted immune response to HAART therapy observed in HIV-HCV co-infected subjects. Direct HCV pathogenicity on lymphocytes may also contribute to lower proliferation of CD4 T lymphocytes. HCV envelope protein (E2) binds to CD81 cellular receptor (98), and HCV has been detected in a number of cells including monocytes, macrophages, B cells, as well as CD4 and CD8 T lymphocytes (210-212).

4.3.2 Relevance

This evaluation is in agreement with other studies. It suggests that CD4 T lymphocyte recovery is blunted following the initiation of HAART. This observation is of obvious clinical significance as poor CD4 T lymphocyte response in the first six months of therapy has been associated with an increased risk of opportunistic infection (213). It may be preferable to first treat HCV infection, achieve a sustained virologic response, and then initiate HAART. This practice could potentially maximize CD4 T lymphocyte recovery. Further, prospective investigation of this phenomenon, and its impact on long term health and survival in HIV-HCV co-infected subjects is planned.

4.4 Limitations

Most of the limitations of this analysis have been addressed through out the Discussion Section. Several additional issues warrant attention.

Individuals selected for this evaluation were HIV-HCV co-infection subjects, initiating and remaining on HAART for at least 6 months. Previous evaluation of HIV-HCV co-infected subjects treated at The Ottawa Hospital Immunodeficiency Clinic demonstrated that only 50% remain on HAART at one year (103). In 40% of cases, hepatotoxicity was responsible for treatment discontinuation. It is possible that the HCV viral kinetics of those subjects developing hepatotoxicity following the initiation of HAART may differ from those in which this adverse event does not occur. Because of this selection bias, the conclusions of this analysis are only applicable to those receiving antiretroviral therapy for at least six months. This was a relatively healthy population. The median CD4 T lymphocyte was greater than 300 cells/μL. None of these subjects had clinical or laboratory evidence of hepatic decompensation. As well, the median baseline transaminase level in this cohort was within normal range. These patients received care at a multidisciplinary clinic with a mandate to provide medical, social, nutritional, and psychological support to HIV-infected individuals. The clinical outcome of patients receiving care in such clinics is superior to that of other HIV clinics. Each of these listed factors should be considered when evaluating the applicability of these study results to those infected with HIV and HCV.

Maximal HIV RNA suppression was defined as less than 500 HIV RNA copies/mL. By current standards, <50 copies/mL would be a more appropriate cutoff. It was necessary to use the higher cutoff because prior to the August 1998, the Roche Amplicor 2.0 System. was utilized for HIV RNA level testing. The lower limit of detection for this assay is 500 HIV RNA copies/ml. After August 98, the Amplicor 3.0 System was used, which has a lower limit of detection of below 50 HIV RNA copies/ml. All but one subject's HIV RNA levels were at least in part performed by the Amplicor 3.0 System. Forty-one of these 49 subjects achieved HIV RNA suppression below 50 HIV RNA copies/ml (and all below 500 HIV RNA copies/ml). This information demonstrates that this cohort was truly "maximally" virologically suppressed.

The lower limit of detection of the Roche Amplicor 2.0 quantitative HCV RNA assay utilized in this study is approximately 600 IU/mL. The qualitative HCV RNA assay (Roche Amplicor) has a lower limit of detection of approximately 50 IU/mL. It is possible that several of the results reported as undetectable by the quantitative system may have been positive by the qualitative assay. Two of these six subjects have been tested by quantitative HCV PCR and found to be negative for HCV RNA. The other four have been lost to follow-up. Unfortunately, limited specimen supply and budget precluded testing with this more sensitive assay on all frozen specimens. The use of the quantitative system likely had no influence on the analysis of $\log_{10}$ HCV RNA change from baseline or CD4 T lymphocyte response. This limitation must be considered when interpreting the analysis of subjects with undetectable HCV RNA levels (Discussion. Part B).

The influence of HCV genotype or the size and direction of change in HCV RNA following the initiation of HAART was not assessed. HCV genotype influences the likelihood of obtaining a sustained virologic response following interferon-based HCV antiviral therapy (215,216). In contrast, HCV genotype does not influence HCV RNA levels over time, the rate of liver fibrosis, or the frequency of long-term complications including cirrhosis and hepatocellular carcinoma (217). A planned prospective study will address this issue (218).

Ethnic background influences the natural history of many diseases. For example, Orientals with chronic viral hepatitis generally present with hepatocellular carcinoma as an endstage disease outcome. Caucasians generally present with decompensated cirrhosis with ascitis, esophageal bleeding, and hepatic encephalopathy. Negroes are less likely to achieve a sustained virological response with interferon-based HCV antiviral therapy than Caucasians. There are no publications evaluating the effect of race or ethnic group on change in HCV RNA following HAART. Forty-seven of the fifty subjects evaluated in this study were Caucasian therefore this factor could not be evaluated. It remains unknown whether ethnic background influences the size and direction of change in HCV RNA level following HAART.

A considerable proportion of this analysis pertains to the influence of alcohol on HCV RNA levels and CD4 T lymphocyte response following the initiation of HAART. Accurate quantitation of alcohol consumption, even when done prospectively, is difficult.

In this retrospective evaluation, each clinic note was evaluated to determine alcohol use. I am confident that in cases where no mention of alcohol use is present within the medical records, excess did not occur and misclassification is unlikely. Likewise, misclassification of excess users of alcohol is unlikely because the average use recorded in the chart records was well above 50 grams of alcohol per day. The actual daily amount and the frequency of consumption are uncertain based on the available information. For the purposes of analysis it was assumed that the amount of alcohol consumption was stable over the entire period of antiretroviral therapy. It is possible that alcohol consumption varied over days, weeks, and months. This uncertainly may have influenced that size, duration, and direction of HCV RNA level and CD4 T lymphocyte count change from baseline. Other studies using standardized quantitation alcohol questionnaires are planned.

Preparation of specimen and duration of frozen storage and may effect quantitation of HCV RNA level. As this was a retrospective study, evaluation of specimen preparation was not possible. HCV RNA level at baseline and months 3, 6, and 12 were not found to correlate with duration of frozen storage suggesting against a significant effect of this variable on these results (data not shown).

As the decision to evaluate CD4 T lymphocyte recovery following the initiation of HAART was made *post hoc*, the results should be viewed as hypothesis generating rather than hypothesis testing (188). With *post hoc* analysis, knowledge of the data may influence the decision to analyze and report certain assays and not others. Although this

is true, the analyses conducted in this study where based on those done in other studies in which the analyses were selected *pre hoc*.

Although this study was sufficiently powered to identify a statistically significant difference in HCV RNA level between baseline and month 3, 6, and 12, the analysis of potential covariates influencing baseline and change from baseline HCV RNA levels was limited by small sample size. The data obtained from this study has been valuable in the design of a prospective study which is powered to evaluate these secondary questions. Although it would have been ideal to include more subjects to increase the power of this analysis, this was not possible. The initiation and maintenance of HAART in the HIV-HCV co-infected population is particularly difficult the due to the increased risk of hepatotoxicity, substance abuse, and the poor average socioeconomic status of this group. Although 236 HIV-HCV co-infected subjects were followed at The Ottawa Hospital-Division of Infectious Diseases Immunodeficiency Clinic between January 1996 and August 2001 only 108 initiated therapy. Of these, only 50 remained on therapy for a least six months, achieved maximal virologic suppress, and had adequate chart and appropriate laboratory specimens available for analysis. Additional sites would have been required in order to increase the sample size. This would have been difficult given issues of limited access to medical records, poor documentation of HIV-HCV co-infection at most clinics. and the time required to review thousands of addition charts to identify appropriate subjects and gather appropriate data. Furthermore, the expense required to run additional quantitative HCV measures was prohibitive. Plans are underway to assess the effect of

HAART on HCV RNA level in a prospective, randomized, multicenter study of 100 HIV-HCV co-infected subjects. Enrollment should begin in the Spring of 2003.

5.0 Conclusion

Antiretroviral therapy achieving maximal virologic suppression resulted in an initial 0.5 $\log_{10}$ increase in HCV RNA level over baseline. From this peak level the HCV RNA level declined toward baseline. In those not consuming access alcohol, the HCV RNA level fell below the baseline at twelve months. This decline in HCV RNA level may be indicative of an improvement in HCV-specific immunity. This may slow the progression of HCV infection and may increase the sustained virologic response rate achieved with interferon and ribavirin antiviral therapy for HCV in the HIV-HCV co-infected population. Long term evaluation of HCV RNA kinetics in HIV-HCV co-infected subjects following the initiation of HAART are required to determine if HCV RNA levels continue to fail below those of baseline and if so, whether there is a point when this decline levels off. Evaluation of markers of HCV-specific immunity that are more specific than HCV RNA level and CD4 and CD8 T lymphocytes will further elucidate the effect of HAART therapy on HCV-specific immunity. This information is relevant as it will confirm current beliefs that potent antiretroviral therapy slows the progression of HCV-related liver fibrosis in HIV-HCV co-infected subjects and provide rational for the practice of differing HCV antiviral therapy until after HIV infection is well controlled.

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7.0 Appendices

7.1 Appendix A

Table A-1

Sample Size Calculation

Delta	Std Deviation	Sample Size (power 0.80)	Sample Size (power 0.90)
.25	1.5	285	381
.30	"	199	265
.35	"	147	195
.40	"	113	150
.45	"	90	119
.50	"	73	97
.55	"	61	81
.60	"	52	68
.65	"	44	58
.70	"	39	51
.75	"	34	45
.50	0.75	20	26
"	1.00	34	45
"	1.25	52	68
"	1.50	73	97
"	1.75	99	131
"	2.00	128	171

7.2 Appendix B

Dichotomous Predictors of Change in HCV RNA Level from Baseline Following Initiation of HAART

Table A-2

Sex as a Predictor of Change in HCV RNA Level

Variable	Month	Category	N	Mean	SD	Median	P
Sex	3	Female	9	0.79	1.65	0.29	0.49
		Male	29	0.48	0.98	0.29	
	6	Female	11	0.18	0.47	0.28	0.51
		Male	39	0.50	1.55	0.22	
	12	Female	4	0.20	0.37	0.20	0.93
		Male	21	0.31	2.45	0	

Table A-3

Change in HCV RNA Level from Baseline as a Function of Protease Inhibitor Inclusion in HAART Regimens

Variable	Month	Category	N	Mean	SD	Median	P Value
Protease Inhibitor	3	Yes	28	0.53	1.01	0.34	0.83
		No	10	0.62	1.55	0.073	
	6	Yes	39	0.52	1.55	0.32	0.37
		No	11	0.094	0.38	0.22	
	12	Yes	19	0.31	2.59	0	0.95
		No	6	0.24	0.21	0.26	

Table A-4

Change in HCV RNA Level as a Function of Baseline ALT Level

Variable	Month	Category	N	Mean	SD	Median	P
ALT	3	< 40U/L	16	0.21	0.32	0.21	0.095
		≥40 U/L	19	0.89	1.55	0.44	
	6	< 40U/L	22	0.38	1.64	0.26	0.73
		≥40 U/L	24	0.53	1.26	0.17	
	12	< 40U/L	15	0.13	2.61	0	0.59
		≥40 U/L	8	0.69	1.82	0.016	

Individuals with consistently negative HCV RNA levels were removed and the analysis repeated.

Table A-5

Change in HCV RNA Level as a Function of Baseline ALT Level for Subjects with Positive HCV RNA Measurements

Variable	Month	Category	N	Mean	SD	Median	P
ALT	3	< 40U/L	16	0.21	0.32	0.21	0.04
		≥40 U/L	15	1.12	1.67	0.53	
	6	< 40U/L	22	0.38	1.64	0.26	0.56
		≥40 U/L	19	0.66	1.39	0.32	
	12	< 40U/L	15	0.13	2.61	0	0.52
		≥40 U/L	6	0.92	2.10	0.75	

7.3 Appendix C

Change in CD4 T Lymphocyte Count from Baseline as a Function of Key Covariates

Table A-6

Correlation Between Change in CD4 T Lymphocyte Count from Baseline and Age at Initiation of HAART

Change CD4 at:	N	Beta	P Value*
Month 1	30	0.10	0.60
Month 3	39	-0.17	0.29
Month 6	49	-0.18	0.22
Month 9	22	0.11	0.63
Month 12	25	0.29	0.15

* 2-sided Pearson Correlation

Table A-7

Correlation Between Change in CD4 T Lymphocyte Count from Baseline and Baseline CD4 T Lymphocyte Count

Change CD4 at:	N	Beta	P Value*
Month 1	30	-0.30	0.11
Month 3	39	-0.01	0.96
Month 6	49	-0.03	0.86
Month 9	22	0.18	0.42
Month 12	25	0.01	0.95

* 2-sided Pearson Correlation

Table A-8

Baseline and Change in CD4 T Lymphocyte Count as a Function of Sex

Variable	Month	Category	N	Mean	SD	Median	P Value
Sex	Baseline	Female	11	396	307	222	0.35
		Male	39	311	247	265	
	1	Female	5	123	162	100	0.41
		Male	25	80	92	63	
	3	Female	9	110	98	87	0.39
		Male	30	81	83	68	
	6	Female	10	150	134	111	0.15
		Male	39	87	122	88	
	9	Female	3	73	85	56	0.42
		Male	19	133	118	115	
	12	Female	3	122	170	180	0.91
		Male	22	131	115	123	

Table A-9

Change in CD4 T Lymphocyte Count from Baseline as a Function of Protease Inhibitor Use in HAART Regimen

Variable	Month	Category	N	Mean	SD	Median	P Value
Protease Inhibitor	1	Yes	24	93	111	74	0.57
		No	6	65	71	48	
	3	Yes	29	87	94	72	0.88
		No	10	92	63	93	
	6	Yes	38	103	130	102	0.71
		No	11	87	111	81	
	9	Yes	16	131	125	114	0.70
		No	6	109	88	117	
	12	Yes	20	143	117	150	0.27
		No	5	76	123	18	

Table A-10

Change in CD4 T Lymphocyte Count from Baseline as a Function of Non-Nucleoside Reverse Transcriptase Inhibitor Use in HAART Regimen

Variable	Month	Category	N	Mean	SD	Median	P Value
NNRTI	1	Yes	7	71	72	63	0.64
		No	23	92	113	74	
	3	Yes	9	90	63	86	0.93
		No	30	87	93	68	
	6	Yes	12	105	126	95	0.87
		No	37	98	127	88	
	9	Yes	5	105	96	130	0.68
		No	17	130	122	112	
	12	Yes	4	133	113	115	0.95
		No	21	129	119	128	

Table A-11

Evaluation of CD4 T Lymphocyte Response to HAART as a Function of HCV PCR Status

Variable	Month	Category	N	Mean	SD	Median	P Value
HCV RNA	1	Non-Detectable	4	109	117	112	0.66
		Positive	26	84	104	71	
	3	Non-Detectable	5	129	126	178	0.26
		Positive	34	82	80	72	
	6	Non-Detectable	6	72	184	75	0.58
		Positive	43	103	118	94	
	9	Non-Detectable	1	115		115	0.93
		Positive	21	125	117	112	
	12	Non-Detectable	3	238	24	249	0.091
		Positive	22	115	119	105	

Table A-12

Change in CD4 T Lymphocyte Count in Relation to IVDU History

Variable	Month	Category	N	Mean	SD	Median	P Value
IVDU	1	Yes	25	80	96	74	0.39
		No	5	124	147	63	
	3	Yes	32	93	90	79	0.45
		No	7	65	70	64	
	6	Yes	40	93	129	102	0.45
		No	9	128	111	88	
	9	Yes	17	134	121	130	0.50
		No	5	93	95	96	
	12	Yes	22	126	124	130	0.69
		No	3	156	82	128	

Table A-13

CD4 T Lymphocyte Increase in Relation to Naïve Versus Experienced Prior Treatment Status

Variable	Month	Category	N	Mean	SD	Median	P Value
ART Experience	Baseline	Naive	21	297	241	253	0.46
		Experienced	29	354	275	300	
	1	Naive	14	120	86	86	0.11
		Experienced	16	59	112	28	
	3	Naive	18	107	91	80	0.20
		Experienced	21	71	80	64	
	6	Naive	20	113	129	110	0.53
		Experienced	29	90	125	81	
	9	Naive	9	191	120	183	0.020
		Experienced	13	79	88	75	
	12	Naive	13	153	130	158	0.31
		Experienced	12	104	104	89	

The change in CD4 T cell count at month 6 was evaluated for the 25 subjects measured at month 12. A similar trend for greater CD4 T cell count increase was observed for these subjects (p=0.233).

Table A-14

Change in CD4 T Lymphocyte Count from Baseline as a Function of Baseline HIV RNA Level

Change CD4 at:	N	Beta	P Value*
Month 1	30	0.35	0.061
Month 3	39	0.18	0.26
Month 6	49	-0.088	0.55
Month 9	22	0.051	0.82
Month 12	25	-0.13	0.53

Table A-15

Change in CD4 T Lymphocyte Count from Baseline in Relation to Baseline HCV RNA Level

Change CD4 at:	N	Beta	P Value*
Month 1	30	0.047	0.81
Month 3	39	-0.14	0.40
Month 6	49	0.18	0.22
Month 9	22	0.18	0.44
Month 12	25	-0.12	0.56

* 2-sided Pearson Correlation

Table A-16

Change in CD4 T Cell Count as a Function of Alcohol Consumption in the Twenty-Five Subjects with Data at Twelve Months

Variable	Month	Category	N	Mean	SD	Median	P Value
Alcohol	1	$\geq$ 50 g /day	6	46	77	10	0.26
		< 50 g /day	11	93	78	68	
	3	$\geq$ 50 g /day	5	70	97	51	0.95
		< 50 g /day	14	72	70	55	
	6	$\geq$ 50 g /day	8	33	105	45	0.13
		< 50 g /day	17	118	134	110	
	9	$\geq$ 50 g /day	6	150	114	180	0.98
		< 50 g /day	11	149	122	115	
	12	$\geq$ 50 g /day	8	120	98	115	0.79
		< 50 g /day	17	134	130	128	

The Ottawa Hospital | L'Hôpital d'Ottawa

Research Ethics Board
Conseil d'éthique en recherches
761-4146 ~ 761-4902 ~ 761-5072
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Friday, August 03, 2001

Dr. Curtis Cooper
Division of Infectious Diseases
Ottawa Hospital - General Campus
501 Smyth Road
Ottawa, ON
K1H 8L6

Dear Dr. Cooper:

Re: Protocol # 2001290-01H **Impact of Highly Active Antiretroviral Therapy (HAART) on Hepatitis C Virus (HCV) Viral Load Levels Over One Year in HIV-HCV Co-Infected Individuals**

Protocol approval valid until - Friday, August 02, 2002

Thank you for your letter dated July 23, 2001. I am pleased to inform you that your study (listed above) was given expedited review by the Ottawa Hospital Research Ethics Board (OHREB) and is approved. No changes, amendments or addenda may be made in the protocol without the OHREB review and approval.

Approximately two months prior to the expiration date listed above, a single renewal form should be sent to the OHREB office.

The Tri-Council Policy Statement requires a greater involvement of the OHREB in studies over the course of their execution. You must maintain as part of your records copies of the signed consent form. As well, you must inform the Board of adverse events encountered during the study, here or elsewhere, or of significant new information which becomes available after the Board review, either of which may impinge on the ethics of continuing the study. The OHREB will review the new information to determine if the protocol should be modified, discontinued, or should continue as originally approved.

Yours sincerely,

Raphael Saginur, M.D.
Chairman
Ottawa Hospital Research Ethics Board