Novel Approaches to Studying Liver Fibrosis

Study Protocol

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I. INTRODUCTION

Since the advent of highly active anti-retroviral therapy (HAART), there have been dramatic reductions in morbidity and mortality from virtually all causes of illness among HIV-infected persons.[1, 2] A glaring exception to this trend is death from endstage liver disease (ESLD). The rates of liver-related deaths have increased from 1.3-13% before HAART to 10-50% in the post-HAART era.[1, 2] In many settings, liver disease is now the leading cause of death among HIV-infected individuals.[3-8] Much of this excess mortality is driven by the growing epidemic of hepatitis C virus (HCV) co-infection. Due to shared routes of transmission, more than 30% of HIV-infected patients are co-infected with HCV in developed countries.[9, 10] HIV-HCV co-infection is also a potentially explosive problem in regions of the developing world with high HCV seroprevalence (5-10%), such as sub-Saharan Africa, Latin America and South-East Asia, which also carry high burdens of HIV.[9]

HCV progresses more rapidly in the context of HIV.[11-13] The reasons for this altered natural history are not completely understood but include immune dysfunction from HIV infection as liver fibrosis has been correlated to lower CD4 cell counts.[14-16] Although HCV-related disease is considered an opportunistic infection that should improve with HAART initiation [17], this has not been clearly demonstrated. Some studies have suggested that regimens containing protease inhibitors (PIs)[18-20] are associated with reduced fibrosis rates, but others have seen no benefit.[21, 22] Although it may be argued that increased HCV-related mortality is simply the "unmasking" of liver disease in individuals who survive longer due to HAART, other factors may be at play including irreversibility of hepatic damage, incomplete immune recovery, chronic hepatotoxicity related to antiretrovirals, and alcohol use. Recent Canadian and international consensus statements have identified the study of the natural history of HCV in the era of HAART and HCV therapy as a top research priority.[23-25]

Fundamental to evaluating questions about the natural history of HCV (with or without HIV co-infection) is to have reliable biomarkers that can distinguish disease states and monitor progression of disease because serial liver biopsies are impractical on a population scale.

Reliable biomarkers could have significant diagnostic and prognostic value, as well as aid in the evaluation of therapeutic responses. Furthermore, biomarker studies may provide a key to understanding why HCV disease progresses more rapidly in the context of HIV. Although many non-invasive markers have been evaluated in HCV mono-infection, and to a lesser extent in co-infection, none has replaced liver biopsy due to poor overall specificity and lack of sensitivity in the early stages of fibrosis.

Given the increasing numbers of co-infected patients and the complexity of this condition, a full understanding of the interaction between these two chronic viral infections is essential. With this aim, I am leading a CIHR-funded, national prospective HIV-HCV cohort study (Canadian Co-infection Cohort Study; CCC) that is actively recruiting 950 co-infected persons. Our primary focus is to determine how HAART impacts liver disease progression. Detailed information on potential confounders, such as drug and alcohol use, access to care and co-morbidities, is collected and we have created a biologic specimen bank (serum, PBMC and liver tissue). Our research team brings together expertise in HIV, hepatology, immunology, public health and epidemiology. We now propose to use the CCC as a platform to carry out in-depth studies aimed at identifying and characterizing biomarkers in HIV-HCV co-infection and to extend beyond the cohort to recruit suitable HCV mono-infected controls. The uniqueness of this proposal is that it is based on a large and extremely well-characterized population-based cohort with extensive follow-up. This will allow the selection of appropriate control groups to account for the multiple potential biologic and social confounders.

II. HYPOTHESES:

Hypothesis 1. Serum biomarkers and/or biomarker patterns will predict the degree of HCV-related fibrosis. These biomarkers will be direct reflections of hepatic injury and thus, will be similar in HCV mono-infection and HIV-HCV co-infection for a given stage of disease.

Hypothesis 2. HIV-HCV co-infected patients will additionally exhibit different serum protein profiles that reflect the accelerated course of fibrosis in this setting and may provide insights into the mechanisms responsible for altered disease progression.

Hypothesis 3. In HIV-HCV co-infection, serum protein expression is differentially affected by immune status (CD4+ T-cell count), degree of HIV replication, HAART exposure, HCV treatment and potentially by other biologic factors (age, sex, HCV genotype) and exposures (alcohol use).

III. BACKGROUND AND SIGNIFICANCE

The burden of HCV infection HCV infection is one of the fastest growing health problems facing industrialized countries with an estimated 170 million persons infected worldwide.[26] An estimated 240,000 (0.8%) Canadians have been infected and most will develop chronic hepatitis.[27] HCV has been compared to a "*viral time bomb"*.[28] Dramatic increases in the rates of cirrhosis, liver failure, transplant needs and hepatocellular carcinoma (HCC) due to HCV are predicted.[29] Over the next decade in Canada, it is predicted that cases of HCV-related cirrhosis will increase by 92%, liver failure and HCC by 126% and 102%, respectively, and liver-related deaths by 126%.[30] Forecasts for annual health care costs in Canada for HCV-related disease range from $103 to $158 million.[31]

HIV-HCV co-infection Worldwide**,** 10 million persons infected with HCV are estimated to be HIV co-infected.[26] Injection drug use (IDU) is currently the main mode of HCV transmission and is a growing risk for HIV infection.[10] Prevalence rates of HCV co-infection among active IDU range from 54% in Toronto to 79% in Victoria.[32] The prevalence of HCV infection in other risk groups is lower but far from negligible, e.g., 4-11.7% in homosexual men.[33, 34]

HIV and the natural history of HCV HIV infection has a strong negative impact on the course of HCV infection. Co-infected individuals progress more rapidly to liver fibrosis, cirrhosis and ESLD compared to those infected with HCV alone.[12, 13, 35, 36] In a meta-analysis, the relative risk (RR) for ESLD in co-infected subjects was 2.92 (95% CI, 1.70-5.01) compared to HCV mono-infected persons.[11] Once cirrhosis develops, there was also a dramatic acceleration to decompensation and death (RR 6.14).[11]

HAART and liver disease Complicating matters, HAART itself may adversely affect HCV-related liver disease. Rapidly evolving cirrhosis with clinical decompensation and even hepatic necrosis have been observed following HAART initiation.[28, 37] Hepatic dysfunction following HAART has been variously attributed to restored anti-HCV responses and to direct hepatotoxicity.[38] Although HCV levels and transaminases return towards baseline after several months of therapy, they still remain abnormally high. Indeed, the true rate of hepatic fibrosis secondary to co-infection and the complex role of HAART in mitigating its progression are not known. Several cross-sectional studies reported lower fibrosis scores among patients receiving HAART.[18-20] Other studies have highlighted the association of antiretrovirals with more advanced fibrosis [39] or have shown no clear benefit of HAART.[21, 22]

HCV treatment Given the potential limitations of HAART, specific treatment of HCV would seem the obvious solution to prevent long-term consequences of co-infection. Unfortunately, in co-infected patients the current standard therapy of pegylated interferon/ribavirin results in relatively poor sustained virologic response rates (SVR; overall < 40%) compared to those with mono-infection.[40, 41] Although genotypes 2,3 achieve better SVR (43-73%) than genotypes 1,4 (29-38%), responses are on average 20% lower in co-infection than in monoinfection regardless of genotype.[42-45] Given the high toxicity of current HCV therapy, it is important to be able to target patients most likely to respond.

In summary, HIV has a negative impact on HCV progression that does not appear to be fully reversed with HAART. Although successful HCV therapy can improve outcomes, only a minority respond to

treatment. Comprehensive longitudinal studies are needed to determine the impact of various interventions on the course of HCV infection. In order to conduct such studies, it is essential to have safe and reliable means of evaluating and following the state of liver fibrosis.

Current problems with evaluating liver fibrosis The development of fibrosis in chronic HCV infection usually follows a progressive course from portal to bridging fibrosis and eventually to cirrhosis. Liver enzymes are poor predictors of the degree of fibrosis and unlike HIV RNA, HCV viremia has no prognostic value. Liver biopsies are therefore the gold standard for staging liver disease and several semiquantitiative scoring systems have been developed [APPENDIX 3]. In addition to the risk, discomfort and expense of this invasive test, sampling variability is a notable limitation of liver biopsy.[46]

Non-invasive markers Because of the need to easily follow patients and intervene prior to the onset of ESLD, non-invasive markers of hepatic fibrosis have been sought by many investigators. Several indirect serum markers have been validated as predictors of significant fibrosis in HCV mono-infection and to a lesser extent in HIV-HCV co-infection. These range from the simplest, AST-to-platelet ratio index (APRI)[47-49] and AST/ALT ratio[50, 51], to those that involve measurement of non-routine laboratory tests or that use complex or proprietary formulas, such as the FIBROTEST.[52, 53] Overall, these models share high levels of sensitivity and positive predictive values (87-100%) for significant fibrosis with area under the receiver operating curves (AUROC) of 0.82-0.89.[54, 55] Unfortunately, they all lack specificity. Overall, serum markers can rule-in or rule-out fibrosis in up to 35% of patients, but cannot differentiate stages of fibrosis reliably.[56] Furthermore, as these are all indirect markers, no pathogenic inferences can be made. Serial measurements of "direct" serum markers such as hyaluronic acid (HA), YKL-40, procollagen III N peptide and cytokines have been shown in limited studies to be associated with fibrosis progression.[57, 58] Of these, HA is the most promising with a sensitivity and specificity for cirrhosis in co-infected patients of 92% and 83%, respectively.[59] Some recent studies suggest that combinations of markers may perform better than individual indices. For example, the SHASTA index combines measurements of AST, albumin and HA (AUROC 0.88 for predicting significant fibrosis in co-infection).[60] Finally, measuring hepatic stiffness (Fibroscan) is promising alone/as an adjunct to serum markers but also does not differentiate stages of fibrosis accurately.[61, 62]

IV. THE CANADIAN HIV-HCV CO-INFECTION COHORT STUDY (CCC)

Building on an FRSQ-funded pilot study, we received CIHR support in 2006 to create the CCC, which is actively recruiting at 13 sites across Canada [see attached Protocol Summary; Summary of Progress]. The primary objective of this study is to determine the effect of HAART on liver disease progression in HCV-HIV co-infection. We are evaluating the contributions of important social factors, toxicities and immunologic factors that may modify fibrosis progression rates. The study is recruiting a total of 950 HIV+ adults with HCV infection. After initial evaluation, follow-up visits occur every 6 months. Medical information is collected using standardized questionnaires. Detailed information on demographics, substance use, risk behaviors and treatment history and blood samples is obtained. Plasma, serum and PBMC samples are stored for future viral, pathogenetic and immunologic studies. We are proposing to use the CCC as a platform to carry out the studies in the present proposal.

Recruitment Statistics [see also: APPENDIX 4]

As of the end of February 2008, 424 patients have been recruited from 11 centres. Two additional sites have recently opened. The average monthly recruitment has been 5 patients/site. With 13 sites, it is anticipated that by September 2008, 875 patients with be enrolled and that cohort will be fully recruited in 8-10 months. There have been 21 deaths and 7 withdrawals from study. Despite the social barriers faced by our participants, only 17 (4%) have been lost to follow-up. *Thus we have clearly demonstrated the feasibility of maintaining a cohort study with a population of patients that is traditionally considered difficult to follow.* There is significant variability in the population to allow for the selection of sufficient numbers in various groups to be able to perform well-controlled studies of potential biomarkers (i.e., 63% on HAART, 10% initiating HAART/year, 20% with liver biopsies).

Validation and application of the APRI to study liver fibrosis in hepatitis co-infection Using data from our cohort, we have demonstrated that a simple model, the APRI[49], is highly predictive of significant fibrosis in HIV/HCV co-infection.[47; see attached Manuscript #1] For significant fibrosis, the AUROC was 0.85±0.06. APRI scores > 1.5 (higher cut-off) were 100% specific and 52% sensitive. In a retrospective cohort study, we subsequently evaluated the evolution of the APRI, determined its predictive value for hepatic outcomes in HIV^+ patients with or without HCV co-infection, and assessed the effect of HAART on the progression of liver fibrosis. A total of 653 HIV^+ patients without liver complications at baseline (540 HIV+ only, 133 HCV^+) were followed over 14 years (median 4.6 years). At baseline, HCV^+ had higher mean APRI compared with HIV^+ (1.01 vs. 0.52, $p < 0.0001$) and APRI changed significantly over time. Baseline APRI was an independent predictor of liver complications (HR: 4.0, 95% CI: 2.5-6.4). Cumulative time on HAART did not protect against liver complications and PI-based HAART was significantly associated with progression of APRI in HCV co-infection and HIV alone.[63; see attached Manuscript #2]

Thus, biomarkers such as the APRI are potentially useful for studying the progression of liver disease, for predicting hepatic complications and for evaluating the effects of HAART longitudinally. However, this simple marker has some important limitations as many factors other than liver disease impact both AST and platelet measures. As an indirect marker, it also provides no insight into the mechanisms that underlie accelerated fibrosis. More direct, specific and sensitive markers for fibrosis are urgently needed.

V. OBJECTIVES

Our overall objective is to identify biomarkers which are predictive of liver fibrosis in chronic HCV-HIV co-infection and that provide prognostic and pathogenic information that may help elucidate the mechanisms underlying accelerated fibrosis in the setting of HIV infection.

Specific Aims:

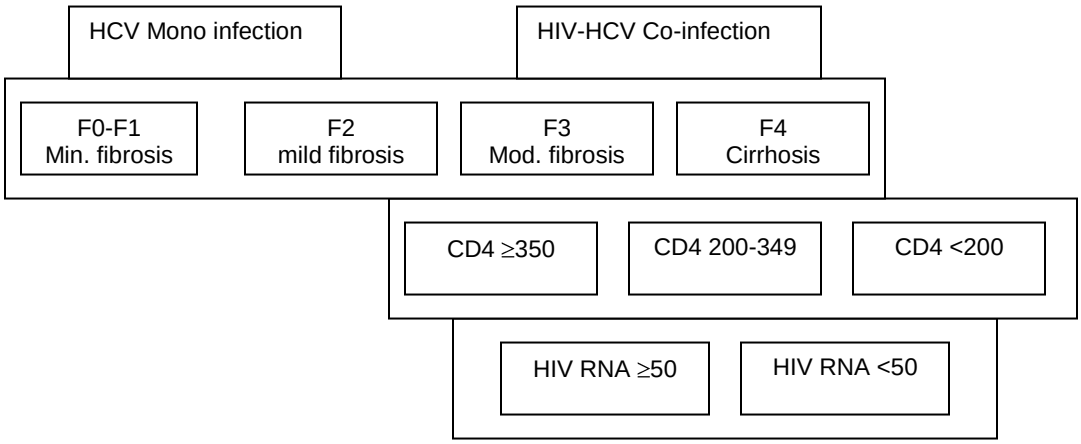
1. To apply high throughput clinical proteomics tools to identify novel serum biomarkers and biomarker patterns associated with fibrosis in HIV-HCV co-infected individuals and HCV mono-infected controls.
2. To validate these biomarkers and calculate the sensitivity, specificity, positive and negative predictive values for the detection of significant hepatic fibrosis compared to liver biopsy (the current gold standard).
3. To compare proteomic profiles in HIV-HCV co-infected and HCV mono-infected controls at similar fibrosis stages to determine if protein profiles are expressed differently.
4. To compare serum biomarkers with ongoing immunologic and non-invasive marker studies.

VI. METHODS

We will use a variety of methods to identify candidate biomarkers that could be 1) predictive of hepatic fibrosis, 2) sensitive to change over time and with exposure to therapeutic interventions, such as HAART, and HCV therapy and 3) potentially informative about the pathogenesis of HCV disease, particularly in the setting of HIV infection. The current proposal forms one of the key elements in a broader research program aimed at identifying markers of fibrosis. Concurrent projects will evaluate immunologic, serologic and radiologic markers (and are/will be funded separately). The novel aspect of the current proposal is the serum proteomics biomarker discovery effort that will be conducted in collaboration with Drs. Brian Ward and Momar Ndao.

Our overall approach for biomarker discovery will proceed step-wise with selection of well-defined controls at each step (Fig. 1). As multiple aliquots of serum and plasma are made at each patient visit, we will be able to evaluate each candidate biomarker in parallel, permitting comparative analyses and assessment of their utility alone and in combination. Furthermore, as PBMC aliquots are similarly stored, comparisons can be made with ongoing immunologic studies at the same time points.

Figure 1. Proposed overall schema for discovery and validation of potential biomarkers.



Biomarker Discovery for HIV–HCV Most biomarker programs focus on a single disease. Our attempt to define serum biomarkers associated with progression of liver disease in HIV–HCV co-infection is quite novel. However, it will likely be complicated by the intrinsic variability of the two underlying conditions and the varied therapeutic interventions used. Although the SELDI TOF MS platform is ideally suited to this type of complex situation, we will still need to exercise great care in selecting both patients and controls (see below). The stages of any biomarker program are Discovery, Validation and Identification. We will approach this complex problem in three general Phases.

Phase 1: Our initial **Discovery** effort will focus on otherwise 'healthy' HCV mono-infected controls and HIV-HCV co-infected patients with preserved CD4 cell counts (>350 cell/ul) and suppressed HIV RNA (< 50 copies/ml for at least 6 months on stable HIV treatment). Both groups will have evidence of replicating HCV (RT-PCR RNA+). Patients with other causes of liver disease or co-morbidities (i.e., current alcohol or drug use, Hepatitis B, TB) or prior/current HCV treatment will be excluded. This strategy should minimize potential "noise" in the serum proteome arising due to HIV-related immunosuppression and comorbidities (see detailed patient selection methods below).

Phase 2: **Validation** studies will be conducted with the groups above but in greater numbers (50-100 per fibrosis category) to confirm the validity of candidate biomarkers and guide prioritization efforts for biomarker **Identification** (see Sample Size Justification below). As samples accrue in our CIHR-funded archive, we will also have the opportunity to apply SELDI analysis to small numbers of serial samples from individual patients (e.g., with repeated biopsies). These sample sets may provide further validation of candidate biomarkers and/or biomarker patterns.

Phase 3: We will expand efforts to discover further useful biomarkers among the lower-abundance proteins and to assess the validity of candidate biomarkers through the study of additional well-defined groups with characteristics that may alter biomarker expression. We will be particularly interested in examining the roles of different levels of HIV-related immunosuppression, HIV replication and HAART exposure as indicated in the algorithm above. We may also evaluate the roles of other confounders in a similar fashion (e.g., alcohol users vs. never users; age, sex and HCV genotypes).

Longterm Plans Ultimately, we plan to evaluate the most promising biochemical, immunologic and proteomic biomarkers longitudinally in the cohort to determine their performance over time and their ability to predict specific clinical outcomes (ESLD and/or death from liver disease) in HIV-HCV co-infection. In addition, we will examine the impact of interventions such as HAART and HCV therapy on these markers.

This proposal will focus primarily on Phases 1 and 2 and is committed to the full identification of all clinically useful biomarkers. We do not believe that spectrometric data, by itself, is particularly interesting (so-called protein profiling, protein fingerprinting). In this program, we hope to identify all

candidate protein/peptide biomarkers both to contribute to the pressing diagnostic/prognostic needs and to gain unique insights into the pathogenesis of HIV-HCV co-infection and its liver complications.

Rationale for the selection of controls As we are primarily interested in studying biomarkers specific for HCV-related liver fibrosis progression, HIV negative, HCV+ controls are important. Although we do not collect samples on HIV negative persons as part of the cohort study, we have established close collaborations with the HCV clinics at the Royal Victoria Hospital (RVH) [see attached Letters from Collaborators]. These clinics serve a large population of HCV mono-infected patients and perform approximately 175 liver biopsies annually. We will prospectively recruit control subjects from these clinics. We have chosen not to include an HCV negative, HIV+ control group initially. Since our primary interest is liver disease and subjects with HIV mono-infection do not routinely undergo liver biopsy (our gold standard tool), we will not initially include an HIV-only group. However, we acknowledge that we may need suitable HIV+ controls in the more advanced phases of this biomarker discovery program as we attempt to tease out the effects of the interactions between HIV and the host from HCV on biomarker expression. Similarly, we will not study HCV negative, HIV negative controls with other liver diseases given that our primary focus is the discovery of biomarkers to distinguish between stages of liver fibrosis in the setting of HCV rather than differentiate HCV related fibrosis from other causes. Some of this latter work is, in fact, being carried out by others (see below).

Proteomics Discovery Program Proteomics as applied to human health research is the direct measurement of the proteome in biological fluids in different disease states. Proteomic studies of chronic HCV infection may permit the detection of proteins produced directly by the virus or of host proteins or peptides uniquely produced and/or processed in the presence of HCV, collectively referred to as biomarkers. Both individual biomarkers and biomarker patterns have the potential for diagnostic and prognostic applications in human health.

Surface-enhanced laser desorption (SELDI)-time-of-flight (TOF) mass spectrometry (MS) SELDI TOF MS is a powerful platform for serum proteomics that is well-suited to population-based studies because several aspects of the analysis can be automated. Within the spectrum of MS technologies, SELDI is a 'top-down' approach, i.e., proteins and peptides are not fragmented prior to analysis. This technology holds promise for development of new diagnostic tests for a wide range of neoplastic [64-68], inflammatory[69-71], and degenerative[72] conditions, as well as chronic infectious diseases[73-75]. In essence, application of SELDI to clinical specimens compares protein profiles in well-matched case and control sera to identify biomarkers that reliably distinguish between the groups/conditions. Useful biomarkers may either be present or absent in case sera vs. controls (i.e., up-regulated or down-regulated). Furthermore, both full-sized proteins and protein fragments that may be subject to unusual or abnormal degradation pathways can be useful biomarkers. Disease-specific biomarker patterns, i.e., SELDI platform-based assays, may be immediately useful to address questions of importance, such as disease progression and response to therapy. Identification of individual biomarkers that discriminate between disease states can provide important insights into the biology of the condition under study and potentially spur the development of 2nd generation, platform-independent assays (e.g. through the development of targeted monoclonal antibodies).

Rationale for SELDI as Screening Strategy The application of SELDI to clinical specimens for diagnostic purposes has not been free from controversy.[76, 77] It is now widely recognized that biomarker discovery programs cannot be based on a single technique or approach (e.g., SELDI, MALDI, LC/MS, pattern recognition, etc.) ***The critical elements of any biomarker discovery program are characterization of the patient population, control over sample handling and strict adherence to standard operating procedures (SOPs)***.[78, 79] Although SELDI has obvious limitations (see below) and vocal detractors, Drs. Ward & Ndao have had considerable success using SELDI as a screening tool in studies of human and veterinary parasitic diseases as well as dengue fever/dengue hemorrhagic fever (see: Summary of Progress). Several other groups have also demonstrated that highly reproducible data can be generated by this approach.[80]

Advantages and Disadvantages of SELDI TOF MS Although current SELDI machines cannot

compete with other proteomic approaches in mass accuracy, this platform has significant advantages in terms of both dynamic range (range of peptide-protein masses that can be detected) as well as automation.[81] The latter characteristic is very important for population-based proteomic studies in which hundreds of samples will be analyzed, as will be the case in the current HIV-HCV program. As a 'top-down' approach, SELDI also has a major advantage in the identification of modified host or microbial proteins (e.g., splicing, glycosylation and phosphorylation variants as well as metabolic byproducts). This characteristic has been very important in Dr. Ward and Ndao's ongoing studies of latent protozoan diseases and acute and complicated dengue infection. Abnormally truncated host proteins are among the most important biomarkers present in subjects with silent Chagas disease [see attached Manuscript #3]. Last, all proteomic techniques that target complex biological fluids, such as serum, suffer from limited penetration into the proteome; low abundance proteins are typically hard to pick out and identify. Like others, Drs. Ward and Ndao use an ever-expanding range of approaches to mitigate this problem, including increasingly sophisticated sample fractionation (see below). Here again, the ease with which SELDI can be automated gives this approach a significant advantage over other techniques since a single sample can generate a large number of individual spectra after extensive fractionation (typically 24-36 per sample in initial discovery efforts). Although we have chosen SELDI as our screening technology, Drs. Ward and Ndao have experience with the full range of proteomic tools, including 2D-DIGE (**Di**fference **G**el **E**lectrophoresis), liquid crystal mass spectrometry (LC/MS) and tandem mass spectrometric micro-sequencing (MS/MS) which may be of use in these studies. Based upon their successes in clinical proteomics to date, they have just received CFI-LOF funding to purchase a dedicated MALDI TOF/TOF mass spectrometer (ABI 4800: **M**atrix-**A**ssisted, **L**aser-**D**esorption and **I**onization) to complement their existing SELDI capabilities (December 2007). This new machinery, including a state-of-the-art robotic sampling platform, will be operational by Spring 2008 and will be adapted to accept a purpose-built cassette (Vermillion Inc) that permits SELDI chips to be analyzed by MS/MS directly. This new infrastructure will be used for parallel validation studies of the HCV biomarkers leading to improvements in confidence, sensitivity and mass accuracy. Critical experiments will be performed using both platforms (individual sera by SELDI, pooled sera by MALDI). This new capacity will also greatly accelerate the speed with which candidate biomarkers can be identified with confidence.

SELDI TOF MS in chronic viral hepatitis Of direct relevance to the current work, SELDI has been applied to predict liver fibrosis and cirrhosis in chronic hepatitis B.[82] Fibrosis indices derived from a proteomic pattern strongly correlated with biopsy scores ($r = 0.83$) and were significantly different among stages of fibrosis. AUROC for predicting significant fibrosis was 0.91 with both 89% specificity and sensitivity. SELDI profiling has recently been applied to HCV mono-infection in which it has shown promise for early detection of heptocellular carcinoma (HCC), as well as studying disease progression and treatment outcomes.[83, 84] When combined with more traditional serum biomarkers (e.g., alpha-feto protein), profiles distinguished cirrhosis and HCC with a reasonably high level of sensitivity and specificity (75% and 92%, respectively).[83] In another study, two SELDI peaks (23/23.5 kDa) were elevated by 50% in the serum of HCC patients and were identified as kappa and lambda immunoglobulin light chains. These peaks distinguished patients with and without HCC with 94% sensitivity and 86% specificity.[85] Multi-peptide/protein patterns have successfully differentiated HCC and fibrosis from patients with F1or F2 fibrosis (80% specificity and 67% sensitivity when applied to random test samples) and further improved when combined with APRI and alpha-feto protein measurements (88% specificity and 82% sensitivity). One of the proteins was identified as apolipoprotein c-I.[86] Most recently, Poynard's group reported that a proteomic index combining 8 peaks provided an AUROC of 0.88 in predicting advanced fibrosis or cirrhosis, significantly greater than their own FIBROTEST.[87] Finally, a recent study of serum protein profiles in HCV mono-infected patients with sustained virologic responses following therapy, identified 37 protein peaks that displayed significant variation during treatment, compared with only one peak in non-responders.[88] Application of an algorithm based on pretreatment serum correctly predicted response to treatment in 89% of

patients (AUROC, 0.92). Although these studies mostly focused almost exclusively on serum 'profiling' and dealt with relatively small numbers of samples and subjects with advanced fibrosis, taken together, they provide an important proof-of-principle for the work proposed herein. To our knowledge, there have been no applications of SELDI proteomics in HIV-HCV co-infection or studies focusing on differentiating earlier stages of fibrosis.

General Methods for Proteomic Analysis using SELDI-TOF MS. [see schema: Figure 2, attached] Quantitative proteomic profiles will be obtained using standard methods.[80] All samples will be analyzed without knowledge of the fibrosis stages [essentially as outlined in Manuscript #3]. Briefly, samples will be thawed on ice at the time of profiling, denatured with 9M urea and fractionated using a range of methodologies. The first 'pass' will typically involve column fractionation with a series of four extraction buffers (pH 9 – organic) and binding to at least two chip chemistries; weak cation exchange (CH10) and immobilized metal affinity capture (IMAC; Ciphergen Biosystems, Fremont CA). At least two different matrices will be applied and spectra will be generated using high- and low-laser intensity (i.e., targeting larger vs. smaller proteins, respectively). Standard QA and AC procedures will be followed throughout. The protein chip arrays will be analyzed using the SELDI PCS4000 Enterprise System (BioRad) normalized using total ion current. Peak clustering will initially be assessed using Biomarker Wizard Software (Vermillion Inc.) at settings that provide a 5% minimum peak threshold, 0.2% mass window, and 2% to 3% signal/noise determination. Intensity values for each peak will be averaged for each duplicate sample pair analyzed and input into BioMarker Patterns software (Vermillion) for classification tree analysis. This decision-analysis software uses a proportion of the generated database to combine these individual biomarkers to achieve the greatest sensitivity and specificity. Using Classification and Regression Trees, multiple biomarkers are correlated with specific phenotypes to improve sensitivity and specificity over single markers. The output is an easy-to-interpret decision tree, using a small panel of markers with defined splitting rules. Each such decision tree represents a potential, 1st generation diagnostic test (a SELDI platform-based assay). The approach outlined above focuses on the identification of potential biomarkers among the higher-abundance proteins and/or their metabolic byproducts (e.g., abnormally synthesized or truncated host proteins). Although the IMAC and CH10 chips typically yield the greatest number of interesting biomarkers, pooled sera from the groups will also be screened over a wider pH range and using additional chip (fractionation) chemistries such as hydrophobic binding (H50), high glycoprotein binding (gadolinium-buffered IMAC) and strong anionic binding (SAX), Any promising candidate biomarkers observed in these parallel screens of pooled serum will be confirmed using individual samples.

Additional sample fractionation to identify lower abundance biomarkers. Given the experience of Drs Ward & Ndao with the blood-borne protozoan diseases and dengue (as well as others who have applied proteomic techniques to samples from subjects with hepatitis: see above), it seems very likely that clinically useful biomarkers will be found among the higher-abundance serum proteins. Such proteins would have obvious advantages in the possible development of second-generation, MS platform-independent assays (i.e. serum concentrations would be high enough to be detected using less sensitive, point-of-care technologies). Nevertheless, it is also very likely that interesting and informative biomarkers will be found among the lower-abundance serum proteins as well. Therefore, we will use pooled sera and a number of strategies, in parallel studies, to seek additional and lower-abundance biomarker proteins in the same patient groups. The search for new ways to identify lower-abundance proteins is evolving very rapidly. Nonetheless, the strategies that we will employ will probably include:

- Pre-fractionation to remove 12-20 of the most common serum proteins (BioRad ProteoMiner™ columns, Brucker ClinProt™ magnetic beads, Invitrogen IgY spin columns). When these common proteins are removed (i.e. albumin, transferrin, immunoglobulin), smaller peaks representing less-abundant proteins are frequently revealed.
- The PF2D (BioRad) can be used to further fractionate complex protein mixtures such as serum. Although no effective concentration occurs, this machinery can permit smaller protein peaks adjacent to abundant proteins to be isolated and identified.

- Size-exclusion spin columns, HPLC or Zoom™ fractionation (Invitrogen) can easily achieve ~5 to 10-fold concentration of targeted biomarkers.

Drs Ward & Ndao have used all of these technologies in their biomarker discovery program with varying degrees of success in different diseases.

VII. RESULTS FROM PILOT STUDIES

Using data and samples collected from the CCC, we performed a limited and unblinded study of four sera from HIV-HCV subjects with advanced fibrosis (liver biopsy showing fibrosis stage 4 within one year of sample collection) compared to four co-infected subjects with minimal-no fibrosis (fibrosis stage 0-1). A summary of the methods and results is shown in **Figure 3**. In part, the intent of this study was to assess sample quality, specifically protein degradation during collection and storage. No sample showed any evidence of degradation. A total of 160 spectra were generated using 6 pH fractions and 2 chip chemistries (CH10, IMAC). After baseline subtraction and manual peak alignment, this preliminary and low-stringency screen revealed a surprising number of possible biomarkers (n = 55) distributed across all fractions and chip types. Compared to the subjects with minimal fibrosis, those with advanced fibrosis had both up-regulated and down-regulated biomarkers (see **Figure 3B**). Even with the limited number of samples included, several candidate biomarkers achieved 'clean' separation between those with advanced vs. minimal fibrosis (e.g.: p-values in the <0.02 to <0.03 range). Although encouraging, these data must be treated with caution because these subjects were selected from the extremes of our population (advanced vs. minimal fibrosis) and the MS operator was not blinded. Furthermore, the Vermillion biomarker detection software that we use (Ciphergen Express™) frequently yields relatively large numbers of candidate markers at the first pass. Based on our experience to date, many of these candidate biomarkers will not be confirmed when larger numbers of samples are run. Nevertheless, several of the biomarkers were of high intensity (e.g. relatively high concentrations are present in serum) and had high molecular weights making them relatively easy to identify using standard technologies. As a result, even these limited data strongly support the strategy we have outlined in this proposal.

VIII. RESEARCH PLAN

Patient Selection To address our first hypothesis, primary analyses will focus on identifying biomarkers associated with fibrosis that are present in both HCV+ and HIV/HCV-co-infected persons. The gold standard for comparison will be the grade of fibrosis assessed on liver biopsy using the Batt and Ludwig scoring system. In this classification, those with minimal portal fibrosis or no fibrosis are scored as F0-F1, periportal fibrosis as F2, bridging fibrosis as F3 and cirrhosis as F4. In order to facilitate recruitment of appropriate subjects, the database of the CCC will be queried. Individuals having undergone a liver biopsy and who have stored serum samples within one year of the biopsy will be selected for study. We will use the fibrosis scores provided by the local site pathologist to select subjects. Standard semi-quantitative scores are used at all centres. The reports from all biopsies performed will be converted to the Batt and Ludwig scoring system for the purposes of analyses [see attached Histologic Grading of Liver Biopsies; APPENDIX 3]. In order to validate the biopsy readings, which may vary due to the expertise of the local pathologist, biopsy slides for all subjects in Phase 1 will be re-read by a single pathologist experienced in liver histology at the McGill University Health Centre. This pathologist will read all biopsies blinded to co-infection status, clinical and proteomic data. HCV mono-infected controls undergoing liver biopsy is part of routine care will be recruited through the RVH Hepatology and Infectious Diseases HCV clinics. Serum samples within one year of liver biopsy will be obtained after informed consent and stored frozen until analyzed.

SELDI Data Analysis-Discovery Previously frozen (–80°C) but never thawed serum samples within one year of liver biopsy will be studied initially. In order to ensure blinding in the SELDI analyses, samples will be aliquoted and coded in Dr. Klein's laboratory and sent to Dr. Ward where SELDI spectra will be collected without knowledge of the fibrosis stage or diagnostic group. Discovery studies (Phase 1) will focus on HIV co-infected and HCV mono-infected groups at the extremes of the range of fibrosis (i.e., advanced fibrosis/cirrhosis (F>3) vs. minimal fibrosis (<F2)). Initially, we will study 10

sera per fibrosis category and diagnostic group (total= 40 patients). In addition to simple across-group and across-category comparisons (ANOVA), correlations between the degree of fibrosis (biopsy scores) and individual biomarker peak intensities will be analyzed by Spearman rank-order correlation. Analyses according to fibrosis stage will be made overall and stratified by HCV-HIV co-infection status. Individual biomarkers that make unusually large contributions to the CART algorithms generated will be targeted for identification at this stage. We will focus on optimal samples for discovery and validation work. Once candidate biomarkers have been validated and identified, we will test intentionally 'mishandled' (e.g., repeated freeze-thaw) to assess the robustness of the each biomarker.

SELDI Data Analysis–Validation Larger numbers of sera will be separated by fibrosis category: minimal fibrosis (F0-1), mild fibrosis (F2), moderate fibrosis (F3), severe fibrosis/cirrhosis (F4) to assess the discriminatory ability of the individual biomarkers or biomarker patterns (n = 50-100 per fibrosis category and infection group). Ideally, equal numbers of patients will be studied in each of these groups. Although it may be more difficult to recruit individuals at the extremes (F0-1, F4), we observed the following distribution of fibrosis scores: 13 (28%) with F0-F1; 14 (35%) with F2, 10 (22%) with F3 and 9 (20%) with F4 among the 46 patients evaluated in our APRI validation study[47]. These data suggest that we should have a fair distribution across stages of fibrosis. If we find that scores are not evenly distributed, we may regroup stages to achieve groups of comparable sizes for analysis. The intensities of individual biomarker peaks and biomarker profiles will be compared within and between mono-infected and co-infected subjects as outlined above. All biomarkers making important contributions to distinguishing between groups and/or fibrosis categories will be confirmed by MALDI TOF MS and identified using a range of immunologic and mass spectrometric techniques (e.g.: EIAs when commercial kits exist or can be built easily, immunoprecipitation, Edmond sequencing, MS/MS).

Data abstraction To assess the contribution of various clinical and biologic data on laboratory markers at the time of serum sampling, data will be abstracted from the CCC database and from the clinical database of HCV mono-infected subjects studied. These values will include but are not limited to demographic and treatment data, HCV genotype, quantitative HCV RNA, ALT, AST, platelet count, INR, total bilirubin, albumin and for HIV infected patients, plasma HIV RNA, CD4 cell count.

Justification for sample size Much of the published protein profiling work does not meet minimal standards for sample size. Although sample size considerations are critical, the process of defining ''adequate'' can be difficult when no prior knowledge exists about the proteins under study. While standard sample size calculations can be applied, none works particularly well in the context of proteomic profiling because so little is known *a priori* about the complexity of the final model before an experiment is completed, or about the individual biomarkers that will be included in that model.[89, 90] This is why biomarker studies are typically divided into three phases. In **Phase 1** (**Discovery**), small numbers of specimens (~10 cases/controls) are subjected to extensive fractionation across multiple chip chemistries and conditions to optimize the detection of discriminatory proteins/peptides. In this phase, the focus is on sensitivity and stringency is typically low. **Phase 2 (Validation)** concentrates on confirmation and consolidation of findings from Phase 1 using much larger numbers of samples to test whether proteins are differentially expressed for each candidate biomarker individually and in various combinations. Sample size estimates depend on the sample variance and desired detectable difference in expression. In previous proteomic studies on HCV-associated HCC described above, mean mass to charge ratios (m/z) of individual proteins detected ranged from approximately 3-25 with standard deviations of 1.3-14.[86, 87] The vast majority, however, have ranged between 2-5 with standard deviations of 2-4. Thus, at the lower range of variance, a sample size of 30 per group would provide 80% power to detect a 1.5-fold difference in protein expression with alpha<0.05. At the higher range of variance, 113 samples per group would be required to provide the same level of power. These estimates are in keeping with the experience of Drs Ward & Ndao and from the wider clinical proteomics literature. In general, 50-100 specimens are required to estimate sensitivity and generate initial specificity estimates in clinical proteomics experiments. In the context of our study, we have planned to study 50-100 subjects per fibrosis stage, in both mono-infected and co-infected subjects (200-400 total

samples). It is in **Phase 3** studies that findings can be further validated through extensive analysis with other sample sets or control groups in addition to protein/peptide identification.

Feasibility Of 424 patients currently enrolled in the CCC, 53% are on HAART (with HIV RNA<50 copies/ml and CD4> 350) of whom 20% have undergone liver biopsy. Thus, we presently have sufficient samples to conduct Phase 1 studies in the co-infected. The full cohort is actively recruiting and is expected to reach its target by December 2008. Extrapolating from the currently enrolled patients, we expect 150-200 eligible subjects available for study. HCV mono-infected will be recruited retrospectively (through database review) and prospectively through the RVH HCV clinics which perform on average ≈175 biopsies/yr. Thus the number of samples we require represents about 40% of available patients.

General Data Analyses Assessment parameters for the accuracy of proteomic biomarkers for the detection of significant hepatic fibrosis and cirrhosis will be calculated as shown in the simplified example in the table below. SAS PROC LOGISTIC (V.8.02; SAS Institute, Cary, NC) will be used to calculate AUROCs. Standard errors for the AUROC will be calculated according to Hanley and McNeil.[91] Primary analyses will group HIV co-infected and mono-infected subjects together according to level of fibrosis. We will also calculate the parameters for HIV co-infected and mono-infected separately. In addition, we will analyze promising candidate biomarkers combined with other markers of fibrosis that we are studying in parallel ongoing projects (e.g. HA, SHATA, APRI etc.).

Table 1. Diagnostic characteristics comparing proteomic biomarkers with liver biopsy scores

		Stage of HCV on Liver Biopsy	
		No Fibrosis/No Cirrhosis	Significant Fibrosis/cirrhosis
Proteomic biomarker	+	a (true positive)	b (false positive)
	-	c (false negative)	d (true negative)

Sensitivity = a/a+c; Specificity = d/b+d; Positive predictive value = a/a+b; Negative predictive value = d/c+d

IX. ROLES OF THE INVESTIGATORS

Dr. Klein is an infectious diseases specialist with expertise in HIV, Hepatitis C, and epidemiology. She has experience in basic and clinical research, cohort studies and randomized trials. Dr. Klein will be primarily responsible for all aspects of the research program including leading the multi-centre CCC as well as recruitment, follow-up and data analysis related to this project. She will be primarily responsible for initiating and reviewing the scientific protocols and overseeing the conduct of the research studies, the manuscripts and presentations. She will be a co-supervisor of all trainees working on the project. As a mentor in the CIHR-sponsored National Canadian Research Training Program in Hepatitis C, she will be able to provide access to a wider HCV training experience for the postdoctoral fellow engaged in these studies. Dr. Ward is past Chief of the McGill Division of Infectious Diseases, Medical Director of the National Reference Center for Parasitology (NRCP) and Associate Director of the Research Institute of the MUHC (Fundamental Science). Dr Ndao is a new faculty member in the Division of Infectious Diseases and Laboratory Director of the NRCP. Dr.Ward and Ndao hold CIHR and CIHR-Industry funding for clinical proteomics studies of human parasitic and viral diseases transmissible by transfusion. They share office and laboratory space that is equipped with the technical expertise and machinery to carry out the proteomic work proposed. Dr Ward recently received a CFI-LOF award to purchase an automated MALDI TOF/TOF mass spectrometer (ABI 4800) that will permit both greater sensitivity and mass accuracy in their biomarker discovery program. As co-investigators, Drs. Ward & Ndao will be directly involved in the design and implementation of these studies, as well as the co-supervision of a post-doctoral fellow. The clinical proteomics consortium that they have built includes Dr B Gibbs (LC/MS and tandem MS/MS at the Sheldon Biotechnology Center, McGill), Dr M Duncan

(2D-DIGE and tandem MS/MS at the U Colorado Proteomics Center, Denver) and the McGill Proteomics Center.

X. EXPECTED RESULTS

Based upon the experience in the Ward/Ndao laboratory, as well as the small but growing literature describing the application of SELDI and related technologies to clinical hepatology, we are confident that the work we propose will reveal new and discriminatory biomarkers in HCV-HIV co-infected subjects. As noted above, many clinical proteomics projects stumble over the issues of access to patients and the collection of adequate patient information. Use of the structured data and sample collection afforded by the HCV-HIV cohort will ensure this is not a problem. Although it is impossible to predict what types of biomarkers will be identified in a discovery program such as this, we anticipate that: a) several biomarkers will need to be combined in order to achieve high sensitivity and specificity for liver disease status. It is certainly possible that the optimal combination will include existing surrogate markers (eg: APRI) as well as new biomarkers discovered in the course of this program and that the optimal biomarker combination may vary with the stage of disease; b) unlike existing scoring systems for liver disease, it is likely that individual serum biomarkers or combinations of biomarkers will be identified that can provide prognostic information rather than simply acting as markers for current disease status at the time of testing; and c) at least some of the new serum biomarkers identified may be informative regarding the underlying immunopathology of HCV-HIV co-infection, giving insights into novel preventative or therapeutic strategies to mitigate disease progression.

XI. STRENGTHS AND LIMITATIONS

This proposal is an example of the type of translational research that can be developed from a population-based cohort study. It provides for a synergistic collaboration between two investigators, combining expertise in clinical study design and statistics with serum proteomic methods. It will serve as an excellent training opportunity for a new generation of HIV-HCV researchers. As discussed above, a major challenge in proteomic studies is that heterogeneity among subjects and the presence of confounding variables can lead to artifacts in the assignment of a phenotypic response to the disease process under study. Failure to consider variations in sample collection, storage and quality can further compromise the ability to identify protein profiles that correspond to specific histologic phenotyes.[92] Thus, the clear advantage to our study is our ability to select samples from a population that is extremely well-characterized, having instituted strict patient selection criteria and rigorous sample collection and storage procedures. We have presented a step-wise discovery program. Phase 2 studies are obviously dependent on success in Phase 1. A growing body of literature supports that the population proteomics approach will be fruitful for studying fibrosis in HCV.[83, 85-88] Indeed, our studies in mono-infected subjects will serve to confirm (refute) work published in this area to date, as well as extend it by identifying all of the candidate biomarkers. Our own pilot data support our ability to detect potential biomarkers that can distinguish advanced fibrosis from minimal to no fibrosis in HIV-HCV co-infection using the techniques we are proposing. We already have sufficient samples to perform Phase 1 studies among HIV-HCV co-infected persons. Our current data support our ability to recruit enough patients for study once the full CCC has been enrolled. Although a considerable number of HCV mono-infected controls must be recruited, we are confident that we can do this rapidly for Phase 1 studies. Given the large number patients evaluated annually in the clinics (n=175), we do not anticipate difficulties in recruiting sufficient controls for Phase 2. However, if recruitment is lower than anticipated, we will extend recruitment to other centres. For example, several CCC co-investigators (e.g., C. Cooper, B. Conway, D. Wong; see collaborators list) run large HCV mono-infection clinics. The advantages and disadvantages of the SELDI platform have been discussed in detail above. Although we have based our primary discovery program on SELDI, we do not intend to rely on a single platform for these studies. The recent acquisition of a high-throughput TOF/TOF by Drs Ward & Ndao further mitigates against both the real and perceived limitations of SELDI.

XI. STUDY TIMELINES

A complete study timeline is provided in the Budget Justification. Briefly, in the first 4-6 months a protocol and consent forms will be prepared and submitted to the IRB for the HCV mono-infected patients. Study personnel will be trained. A data analyst will query the CCC database and link to sample repository for Phase 1 studies, as well as to create a database for HCV mono-infected study data and samples. We will recruit a post-doctoral health professional. The fellow will receive training in SELDI and proteomic techniques. HCV mono-infected controls will be recruited and phase 1 studies will be initiated by the end of the first year of the grant. In year 2, recruitment of HIV-HCV cohort participants and HCV controls for Phase 2 studies will continue. Analytic methods will be optimized and Phase 1 studies will be completed and analyzed. In the third year, Phase 2 studies will be performed and analyzed. Results will be presented at scientific meetings. Finally, in the last 6 months, we will plan future validation (Phase 3) studies and submit final results for publication. We envisage that protein identification efforts will begin by the end of the first year and continue through the second and third years of the study as new, discriminatory biomarkers are discovered among the lower abundance proteins.

XII. IMPLICATIONS AND FUTURE DIRECTIONS

While liver biopsy is currently accepted as the gold standard for evaluating cirrhosis, it is an imperfect one. The identification of protein biomarkers that reliably predict stages of fibrosis will have important implications for the study and care of HIV-HCV co-infected persons. By comparing HCV mono-infected with HIV-HCV co-infected persons we also hope to discover proteomic differences which will shed light on the pathogenesis of the accelerated disease course in HIV co-infected persons. If we are successful in this discovery phase, more detailed evaluations of the roles of different levels of immunity and HIV replication, as well as the roles of potential co-factors in driving fibrosis progression, will be explored. Furthermore, it will be important to understand if the proteomic biomarkers we identify are correlated with other described indirect and direct fibrosis markers and if proteomic markers are superior to or synergistic with these markers in predicting fibrosis. With multiple aliquots of serum and PBMCs, we are in a position to evaluate a wide range of biomarkers in parallel. In ongoing and planned studies, we will focus on studying various indirect (e.g., APRI, FIB-4) and direct (e.g., hyaluronic acid) markers in evaluating and following hepatic fibrosis. In addition, the Hepatology group at the RVH is acquiring a Fibroscan machine. We will be participating in validation studies in HCV mono-infected and co-infected patients and plan to co-enroll the subjects captured in the proteomics study. Our long-term goal is to identify candidate biomarkers that could be used longitudinally to study liver disease progression in population-based cohorts and eventually in the clinical setting. Specifically, we hope to prospectively determine their predictive value for clinical outcomes (e.g., ESLD and/or death from liver disease in HIV-HCV co-infection) and to study the role of biomarkers in assessing the effects of HAART and HCV therapy on liver fibrosis progression, accounting for other potential biologic and social factors. The wealth of longitudinal clinical and laboratory information and samples collected as part of the CCC places us in a unique position to realize these objectives in a well-characterized population.

XII. CONCLUSIONS.

The increased rates of ESLD and death among HIV/HCV co-infected persons have important implications for healthcare resources. The substantial health and economic gains thus far achieved by HAART will likely be increasingly tempered by the growing number of co-infected individuals requiring care. In order to address this challenge, a greater understanding of the impact of co-infection on morbidity and mortality, the effects of HAART on modifying disease progression and the people who will most benefit from targeted interventions is essential to stemming the epidemic. The discovery of reliable biomarkers of hepatic fibrosis will have many potential benefits including: reducing need for liver biopsy, facilitating follow-up and timing of HCV treatment, and evaluating the impact of HAART and other factors on liver disease progression. The discovery of biomarkers that reflect treatment responses would represent a major contribution that would have both research and clinical implications. Finally, a better understanding of this process may permit interventions to prevent or slow this devastating complication.

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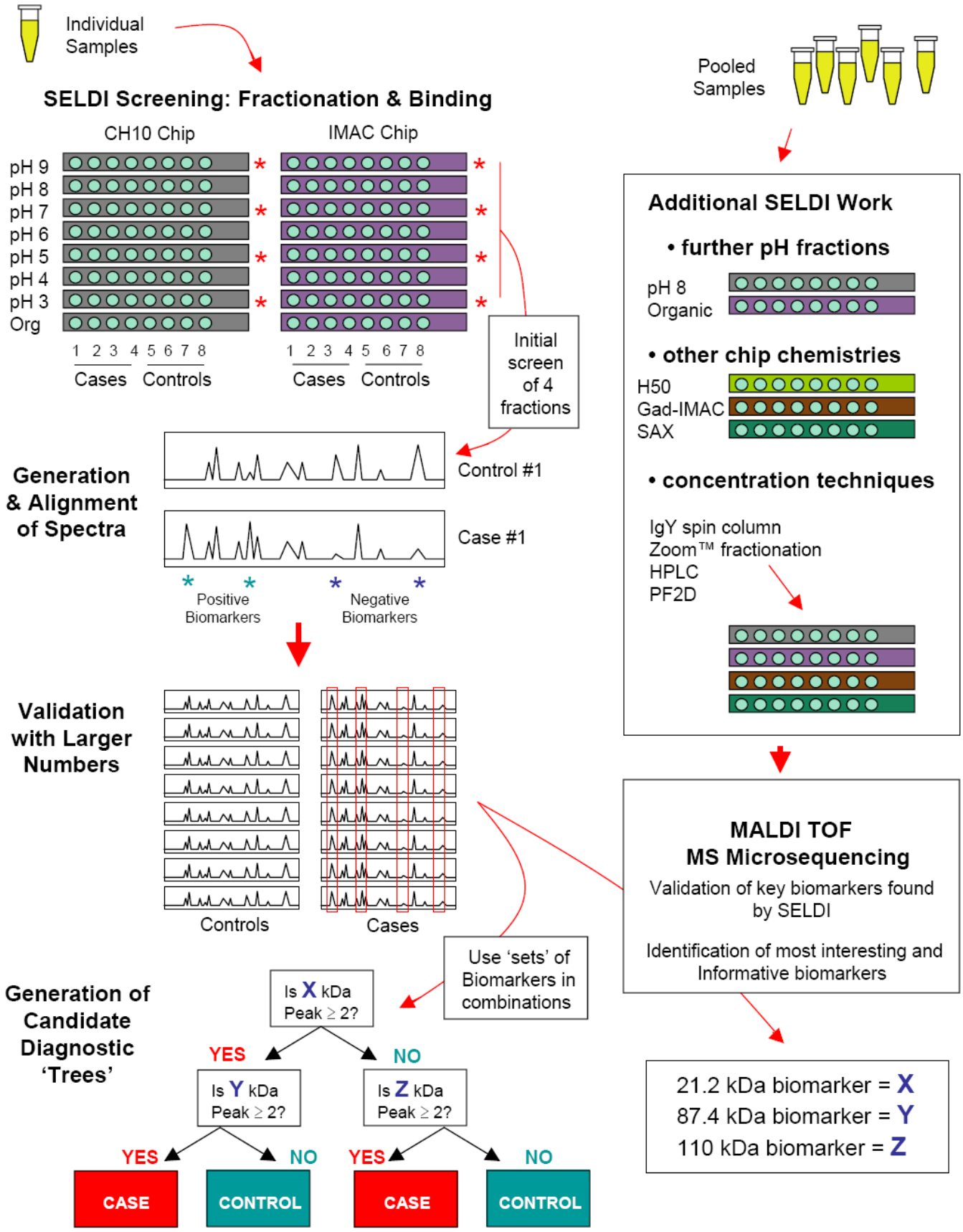
APPENDICES TO RESEARCH PROPOSAL

1. **Figure 2.** Clinical Proteomics: Sample Handling
2. **Figure 3.** Pilot Studies :Summary of Advanced Fibrosis vs. Little to No Fibrosis
3. **Figure 4.** Semiquantitative Scoring Systems for Histologic Grading of Liver Biopsies
4. HIV-HCV Cohort Site Recruitment Statistics

APPENDIX 1.
Figure 2. Clinical Proteomics: Sample Handling



APPENDIX 2

Figure 3. Pilot Studies: Summary of Advanced Fibrosis vs. Little to No Fibrosis

A. Summary of Fibrosis vs Little to No Fibrosis

M/Z range of Candidate Biomarkers: 2 - 190 kDa

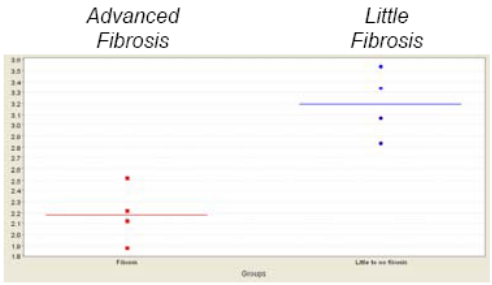
Chip type / Fraction	CM10	IMAC30	Total # of biomarkers
F1- pH9	3	9	12
F2- pH7	2	3	5
F3- pH5	8	7	15
F4- pH4	5	2	7
F5- pH3	5	4	9
F6- Organic	5	2	7

B

F4CSH – 17284 Da

(*p-value = 0.02*)

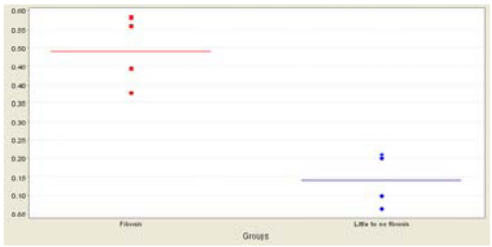
Down-regulated in subjects with advanced fibrosis (dot-plot)



F1ISH – 27,668 Da

(*p-value = 0.02*)

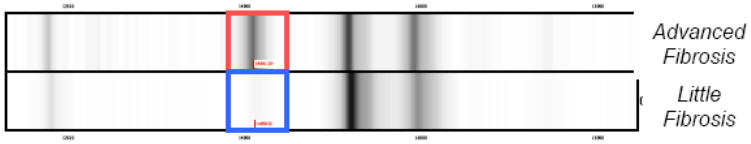
Up-regulated in subjects with advanced fibrosis



F3CSL – 14,063 Da

(*p-value = 0.03*)

Up-regulated in subjects with advanced fibrosis ('gel-view' of spectra)



Individual sera from 4 HIV-HCV subjects with advanced fibrosis and 4 co-infected subjects with minimal fibrosis were fractionated and bound to CH10 and IMAC ProteinChips™. As a preliminary screen for sample quality and candidate biomarkers, 5 fractions were assessed using both low and high laser intensity (revealing smaller vs larger proteins respectively). At a fairly low stringency (p<.05), these 160 spectra generated revealed a total of 55 candidate biomarkers (see A). Although many of these possible biomarkers (particularly those of low intensity) will not be confirmed when larger numbers of samples are analyzed, several of the high intensity markers show quite good separation between the subjects with advanced liver disease vs earlier-stage fibrosis See examples in B. The nomenclature used is (F = fraction as per Section A, chip type is either C (CH10) or I (MAC), matrix used in all cases was sinapinic acid (S) and laser intensity is indicated by L (low) or H (high)).

APPENDIX 3.

Figure 3. Semiquantitative Scoring Systems for Histologic Grading of Liver Biopsies

METAVIR algorithm for grading activity in chronic hepatitis

Piecemeal Necrosis	Lobular Necrosis	Activity Score
0	0	A0
0	1	A1
0	2	A2
1	0	A1
1	1	A1
1	2	A2
2	0	A2
2	1	A2
2	2	A3
3	0,1,2	A3

Piecemeal necrosis:

0 None
1 Focal, some portal tracts
2 Focal all, or diffuse some portal tracts
3 Diffuse, all portal tracts

Lobular necrosis:

0 < one necroinflammatory focus per lobule
1 At least one necroinflammatory focus/lobule
2 Several foci per lobule or bridging necrosis

Bedossa, P. and METAVIR Study Group, Hepatology 1996;24:289-93

Comparison of fibrosis staging systems for use in chronic hepatitis

Fibrosis	Scheuer	Metavir	Batts-Ludwig	Ishak
None	0	0	0	0
Portal (few)	1	1	1	1
Portal (most)	1	1	1	2
Periportal ± rare portal-portal septa	2	1	2	2
Few bridges or septa	3	2	3	3
Numerous bridges or septa	3	3	3	4
Incomplete cirrhosis	4	4	4	5
Cirrhosis, definite or probable	4	4	4	6

APPENDIX 4.
HIV-HCV Cohort Site Recruitment Statistics

Clinic Site	Total HCV pts followed	Number expected to recruit	Number Recruited to Date (Feb 2008)	% With biopsy
MCI	150	120	131	16
MGH	120	80	45	10
NDH/ CHUM	140	100	130	15
QL/EM	200	150	11	10
Ottawa	300	150	20	20
Toronto	87	60	24	52
Hamilton	100	50	0	60
Calgary	143	100	37	12
Vancouver	350	150	20	5
Halifax	57	50	2	10
Windsor	30	20	4	5
TOTAL	**1677**	**1030**	**424**	**20**