SHARED - **S**urveillance of **H**epatitis C **A**ntiviral **R**esistance, **E**pidemiology and Metho**D**ologies

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Collaborative HCV Resistance Database

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Study Design

Non-interventional, multicentre cohort study, 4 years subject to renewal.

A. SUMMARY

The advent of interferon-free direct-acting antivirals (DAAs) has delivered unprecedented sustained viral response (SVR) rates of >90% in genotype (GT) 1 & 4 patients chronically infected with hepatitis C virus (HCV). In patients who fail to achieve SVR, resistance–associated substitutions (RASs) are often selected. This problem is particularly notable in genotype 3 and treatment experienced GT 1a patients where SVR rates are

lower (85-90%), and for patients who previously failed a regimen including a NS5A-inhibitor, in whom RAVs are detected in nearly 100% of cases. (*1-3*). The presence of pre-treatment/baseline RAVs is associated with reduced SVR in patients infected with GT 1a & 3 treated with NS5A class of inhibitors. Also host factors, prior treatment history and liver fibrosis all appear to impact the rate of SVR. The interplay between RAVs and host/disease parameters and their combined effect on treatment outcomes have not been fully appreciated. Given that 5-15% (depending on genotypes) of DAA-treated patients will fail therapy and a substantial portion of these patients harbor RAVs, transmission of resistant viruses poses a threat to public health.

Studies of HCV antiviral resistance have been limited by the small sample size of virologic failures due to high SVR rates. In studies using DAA regimens, there were no consistent criteria used to determine which RASs should be included in analyses and no standardization of analytical methods. The heterogeneity of resistance reporting has made interpretations difficult and significantly hampered the use of this information in guiding clinical decision-making. For HIV, the HIV Drug Resistance Database sponsored by Stanford University has provided comprehensive information for correlating virus mutations with clinical outcomes of antiretroviral treatments. This information has been widely used by clinicians in designing treatment strategies. A similar database has not yet been established for HCV.

We propose to build an HCV Drug Resistance Database by collecting HCV sequences and clinical data through international collaborations, thus including a large number of patients from diverse clinical settings who have been treated with DAAs. Our goal is to conduct in-depth data analyses, generate a clinically relevant interpretation system for HCV antiviral resistance, and translate the knowledge in HCV resistance into more effective treatment options, especially for DAA-experienced patients. In this project, we seek to obtain quality HCV sequences that are linked with well-characterized clinical information to derive genotype-treatment correlations, genotype-phenotype correlations, and genotype-clinical correlations. There are 5 research objectives in this Collaboration: (1) collect HCV sequences from virologic failures, conduct resistance analysis, define the role of known RASs, and evaluate novel mutations not previously described; (2) evaluate the impact of baseline HCV polymorphisms on treatment outcomes; (3) report the *in vitro* drug susceptibility of mutations selected by approved DAAs and establish biological cut-offs to predict clinical outcomes; (4) describe the prevalence of genetic polymorphisms in HCV genotypes 4-6 and their distribution in different geographic regions; and (5) share technologies and know-how for resistance evaluation. High-quality research using tools in biostatistics, epidemiology, bioinformatics and technologies coupled with virology and clinical expertise will be used for the data analyses.

B. BACKGROUND

B1. Global Distribution of HCV

Hepatitis C infection is a leading cause of liver diseases, liver cirrhosis, and hepatocellular carcinoma. Globally, over 170 million people are infected with hepatitis C, and ~71 million (95% CI 62.5 – 79.4) of individuals have viraemic infections in 2015.(*4, 5*) Between 1990-2013, global viral hepatitis death increased from 0.89 million [UI 0.86

- 0.94] to 1.4 million [UI 1.38-1.54]. (*6*) The viraemic prevalence of HCV varies by regions: ≤1% in North America, central/western Europe, Australia, Latin America, Carribean, Oceania, central/east/south Africa, and high-income Asia-Pacific countries (e.g. Japan, Singapore, South Korea); and 1 – 2% in east/south/southeast Asia, eastern Europe and west Sub-Saharan Africa. High endemic countries such as central Asia, Egypt, Pakistan, Syria, Gabon, Georgia, Mongolia and Uzbekistan have a prevalence of 3.5 – 7%.(*4*) The most frequent mode of transmission in developed countries is through sharing drug-injection equipment, whereas unsafe injection and health care exposures through poor infection control practices are the predominant routes of transmission in low-mid income countries with high prevalence of hepatitis C.

The etiologic agent for hepatitis C infection, HCV, has diverse sequences which can be classified into 7 genotypes (GTs) and 84 confirmed subtypes based on the phylogenetic and sequence analyses of the viral genome.(*7*) The nucleotide sites within the HCV genome may vary up to 50% among strains from different genotypes. Genotype 1, which is responsible for the largest number of all HCV infections (83.4 million, 46.2%), represents the predominant genotype worldwide. Genotype 3, the second most common infecting genotype (54.3 million, 30.1%), is prevalent in South Asia, Russia, Australia and many West European countries. Genotype 3 constitutes approximately 20-30% of the HCV infections in Canada and the United States. Genotypes 2, 4 and 6, which are responsible for the remaining 25% of the global HCV infections, are most frequently found in West/Central/North Africa and East/Southeast Asia, respectively.(*8*) Less than 1% of all HCV infections are attributed to GT 5, which are predominantly found in South Africa.

B2. HCV Treatment

The treatment landscape for HCV infection has undergone a remarkable transformation in the last decade. Several DAAs specifically targeting different viral proteins have been developed (Table 1). Fixed-dose combination regimens containing 2-3 classes of DAAs have now replaced the traditional standard of care using pegylated interferon (PEG-IFN) and ribavirin (RBV). These interferon-free DAA regimens have delivered unprecedented SVR rates of >90% in GT 1 patients chronically infected with HCV. Lower response rates (85 – 95%), however, are observed in GT 2 and 3 patients, in particular in those who are presented with liver cirrhosis or have previously failed PEG-IFN and RBV therapies.(*1-3*) Although Harvoni™ (LDV/SOF) and Epclusa™ (VEL/SOF) are approved for the treatment of GTs 4, 5 and 6 in the United States and Europe because of the high unmet medical needs, the registration of these regimens was primarily based on studies of very few patients (<127, <41 and <45 patients for GT4, 5 and 6, respectively).(*9, 10*) The efficacy and resistance profiles of these regimens in these genotypes in a large population and real world settings deserve further investigation.

Table 1. Approved Direct Acting Antivirals for HCV Infections

NS3 Protease Inhibitor	NS5A Inhibitor	NS5B Polymerase Inhibitor
boceprevir (BOC)	daclatasvir (DCV),	sofosbuvir (SOF)

telaprevir (TVR)	ledipasvir (LDV),	dasabuvir (DAS)
simeprevir (SMV),	ombitasvir (OMB),	
paritaprevir/ritonavir (PAR/r),	elbasvir (EBR)	
grazoprevir (GZR)	velpatasvir (VEL)	
asunaprevir (ASV)	pibrentasvir (PIB)	
vaniprevir (VAN)		
glecaprevir (GLP)		
voxilaprevir (VOX)		

B3. Resistance-Associated Substitutions in Virologic Failures After DAA Therapy
Hepatitis C virus exists as a quasispecies within an infected host. Continued viral replication in the face of sub-optimal drug pressure during DAA treatment may result in the selection of drug-resistant viruses, or accumulation of further mutations that reduce drug susceptibility or provide fitness to the drug-resistant variants. As a result, patients who fail to achieve SVR often have detectable RASs. In an integrated analysis of the frequency of post-treatment RASs in patients who experienced virologic failure after LDV/SOF therapy, 71.4% (30/42) of the GT1a and 88.9% (8/9) of the GT1b-infected patients had detectable NS5A substitutions at viral rebound. The frequency of these substitutions in NS5B was lower than in NS5A; 7.3% (3/41) in GT1a and none in GT1b.(*11*)

The association of RASs was universally associated in subjects who failed a combination regimen containing a NS3 protease inhibitor and an NS5A inhibitor, such as GZR/EBR. In a combined analysis from 7 clinical trials, 100% (37/37) GT1a, 100% (8/8) GT1b and 100% (5/5) GT4-infected patients had at least one resistance-associated NS3 or NS5A substitutions at the time of virologic failure.(*12*) Of note, the frequencies of RASs (~80%) in NS3 and NS5A are similar suggesting that drugs targeting NS3 or NS5A are susceptible to RAS selection and that resistance to both the NS3 protease and NS5A class inhibitors classes can coexist in subjects who failed such regimens.(*12*)

Although improving the barrier to resistance is achievable in regimens that contain multiple DAAs, patients who fail such regimens are still vulnerable to antiviral resistance selection. Viekira Pak is a regimen consisting of 3 classes of DAAs: PAR/r (a protease inhibitor), OMV (an NS5A inhibitor), and DAS (an NS5B inhibitor). In a pooled analysis of subjects treated with PAR/r/OMV/DAS with or without ribavirin (RBV) for 12 or 24 weeks, 88% (51/58), 78% (45/58) and 67% (38/58) of the GT1a subjects had treatment-emergent NS3, NS5A and NS5B mutations, respectively, at viral rebound. Notably, 53% of the virologic failures had RASs from all 3 classes of DAAs.(*13*)

Although DAA therapies have significantly improved the SVR rates, antiviral resistance selection in subjects who fail the treatment is almost certain. Several pan-genotypic regimens containing 2-3 classes of DAAs with improved resistance profile are currently under clinical development. It will be some years before these regimens can be universally rolled out, and to a low cost in developing countries as generic drugs.. Given

the 5-10% failure rates of the currently approved DAA regimens, a significant number of patients in the global HCV-infected population will have antiviral resistance. Prevention of onward transmission of resistant HCV, as well as rescue treatment strategies for patients who failed the first line therapies, are needed.

B4. The Impact of Baseline RASs on Treatment Outcomes

The intra-host circulating HCV displays an extremely high genetic heterogeneity due to the lack of a proof-reading mechanism in the viral polymerase. It is estimated that about 2.5 x 10^{-5} mutations per nucleotide per genome replication occurs *in vivo*. With the genome size of 10 kb and a high turnover rate of 10^{10}-10^{13} virions/day, variants with reasonably good fitness can be selected during the chronic HCV infection.(*14, 15*) Under suboptimal drug concentrations, some of these drug-resistant variants are rapidly enriched leading to viral breakthrough during treatment or relapse after treatment.

The impact of baseline drug-resistant HCV variants on treatment outcome was first documented in patients treated with SIM in combination with PEG-IFN/RBV. Subjects infected with HCV GT 1a having a Q80K substitution within NS3 have been shown to have lower SVR rates than those without (47-58% vs. 79-84%).(*16, 17*) Q80K is a genetic polymorphism found mainly in subjects infected with HCV GT1a but seldom in GT1b. The prevalence of Q80K in Europe varies geographically ranging from 4.8% in Norway to as high as 75% in Poland.(*18*) In Canada and the United States, the prevalence of Q80K is estimated to be 47% and 34%, respectively.(*18, 19*). Since Dec 2015, AASLD guidelines (www.hcvguidelines.org) recommend monitoring Q80K for interferon-treatment-experienced GT1a patients with cirrhosis when considering treatment with SMV plus SOF.

Reduced response associated with baseline RASs is also seen in several newer DAA regimens. In treatment-naïve subjects or prior PEG-IFN/RBV relapsers who received 12 weeks of GZR/EBR, the presence of baseline EBR RASs reduced SVR rates from 98% to 58%.(*20*) The effect was even more dramatic in the cohort of patients who had a null response to PEG-IFN/RBV; 29% vs. 97% SVR were observed in subjects with and without baseline RASs, respectively. The impact of baseline RASs diminished with an addition of RBV or an extension of treatment duration to 16 weeks.(*21*)

Sofosbuvir is a nucleotide prodrug targeting the HCV NS5B polymerase. Substitutions resistant to SOF were rarely detected in the clinic due to impaired viral replication capacity. SOF-based combination regimens such as Harvoni™ (LDV/SOF), Epclusa™ (VEL/SOF) or DCV/SOF, in theory, should give a higher barrier to resistance than regimens containing other DAA classes. In a study of 1566 patients treated with guideline-recommended LDV/SOF, GT1a patients who harbored baseline NS5A RASs experienced a 9 - 12% reduction in SVR rates.(*22*) A similar reduction in SVR rate was seen in GT1b patients.(*23*) Further, the second generation combination regimen, VEL/SOF, although it provided improved response in GT1 patients, the presence of baseline RASs in GT3 still had a significantly reduced treatment response: 84-88% SVR in patients with baseline RASs versus 97% in those without RASs.(*19*) In

decompensated cirrhotic patients, the SVR rate in the presence of the baseline Y93H RAS was 50%.(*24*)

It is estimated that the prevalence of RASs in NS5A inhibitors is about 8-16% (with 15% sequencing cut-off).(*23*) At 10–40% failure rates for those who have baseline RASs, millions of HCV patients will likely experience virologic failure from DAA therapy. This preventable risk has led to the recommendation of resistance testing in selected populations by both the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of Liver Diseases (EASL).(*25, 26*) Identification of baseline RASs can help to optimize treatment strategies as demonstrated in the study with GZR/EBR.(*21*)

B5. Treatment Options after DAA Failure

The results from the above studies have arguably demonstrated that there is a significant association of baseline RAVs with reduced SVR, and that selection of treatment emergent RAVs is almost certain in subjects who fail DAA therapies. The longevity of RASs varies by drug classes. The median time to loss of NS3 RASs ranges from 6–14 months based on population sequencing (*27, 28*), whereas a much longer time is required to lose the NS5A RASs. At 92 weeks following virologic failure, 82% of the patients still had detectable NS5A RASs.(*29*) Given that DAAs within the same class, in general, have similar resistance profiles (i.e. cross-resistant to each other) and that most NS5A RASs persist after treatment, it presents a significant challenge for coming up with a rescue treatment option for those who failed DAA therapies. Also, onward transmission of drug resistant viruses will present a serious threat to public health.(*30*) (*31*)

Several studies were conducted to explore rescue treatment options among subjects who had failed DAA-containing regimens. One strategy was to treat patients with different classes of DAAs that had no overlapping resistance profile, or a second generation DAA with an improved resistance profile. For prior NS3 protease inhibitor treatment failures, the second line regimens could be LDV/SOF, DCV/SOF or GZR/EBR, which in general yield >90% SVR although the response from the GZR/EBR regimen was markedly compromised by the presence of pre-treatment NS3 RAVs.(*32, 33*) For prior NS5A inhibitor treatment failures, however, the main option with the currently approved regimens is to retreat these patients for an extended duration and/or to add RBV or SOF. Depending on the pre-treatment characteristics of the patients, SVR rates from 70% to >90% have been observed in different studies using these strategies.(*34-36*) It is worth mentioning that only a fraction of the patients in these studies had detectable RASs before retreatment, and the majority of patients had either received a short course (4 – 8 weeks) of DAA regimens or received a lower than the recommended dose in previous treatments.

Recently, newer therapies containing triple DAAs or second generation doublets have yielded encouraging results. In a phase 3 study,263 NS5A inhibitor-experienced patients were treated with 12 weeks of VEL/SOF/VOX, 91–97% SVR rates were observed in GT 1–6 patients with or without cirrhosis.(*37, 38*) Among these patients, 97% had baseline NS3, NS5A or NS3+NS5A RASs. Similarly, interim results of another triple combination

regimen, MK-3682/GZR/RZR, in GT1 patients administered for 16 weeks plus ribavirin or 24 week without RBV led to 98-100% of patients with viral suppression 8 weeks after completion of treatment. In this study, 4% of the patients had ≥ 3 NS5A RAVs, and 55% had dual NS5A and NS3 RAVs.(*39*) Double combination of glecaprevir (ABT-493)/pibrentasvir (ABT-530) led to comparable results in GT1 non-cirrhotic patients.(*40*)

B6. Limitations in the Current HCV Resistance Evaluation

Most strains of HCV do not infect tissue culture; thus antiviral activity is often assessed using HCV replicons in tissue culture. An HCV replicon is a sub-genomic HCV RNA that contains all of the non-structural HCV proteins (e.g. NS3, NS5A, and NS5B) without the structural viral proteins. Drug susceptibility, reported as EC_{50}, is defined as the effective drug concentration that inhibits 50% of viral replication. Phenotypic resistance to any particular drug is expressed as EC_{50} fold-change (FC),which is the ratio between the EC_{50} against the mutant and the wild-type replicons; the higher the EC50 FC, the greater the resistance. Until recently, *in vitro* evaluation of substitutions has been restricted to GT1a and 1b. Consequently, the phenotypic characterization of non-GT1 RASs is poorly understood. Since *in vitro* evaluation of resistance for clinical isolates is not feasible in HCV, biological and clinical cut-offs for HCV RASs cannot be readily determined.(*41*) The paucity of phenotypic data and the lack of clarity on resistance interpretation remains a major challenge for wide scale clinical use of HCV resistance testing.

Genotypic resistance evaluation is commonly conducted by isolating the virus from blood and sequencing the HCV genome using population-based Sanger sequencing, which has a limit of detection of ~15-20% for viral variants. Over the last decade, Next-Generation Sequencing(NGS) has gained increasing popularity in resistance testing. Next generation sequencing offers unprecedented throughput, scalability, speed, and depth of information compared to traditional Sanger population sequencing.(*42*) NGS can be used to identify minority variants as low as 1%. Although most clinical studies use 15% as a cut-off for variant determination, resistant variants existing at lower levels may play a major role during virologic failure.

Clinically, studies of HCV antiviral resistance have been limited by the small sample size of virologic failures, due to high SVR rates. In studies where HCV resistance was assessed, there were no consistent criteria used to determine which RASs should be included in the analyses and no standardization of analytical methods. The heterogeneity of the resistance reporting has made interpretation challenging and has significantly hampered the use of this information in guiding clinical decision-making. Furthermore, most of the resistance information available to-date has primarily been focused on GT 1; while information for GT 2, 4 - 6 is practically non-existent. GT 2, 4 - 6 are common in resource-limited countries where the prevalence of HCV infection is high, and knowledge in drug resistance is lacking.

In HIV, the HIV Drug Resistance Database sponsored by Stanford University has provided comprehensive information for correlating virus mutations with clinical outcomes of antiretroviral treatments. This information has been widely used by

clinicians in designing treatment strategies. A similar database has not yet been established for HCV.

C. STUDY AIMS AND OBJECTIVES

C1. Aims

The purpose of the SHARED initiative is to create a merged dataset from cohorts of well-characterized HCV sequences through international collaborations. The HCV sequences will be linked with patient information, disease characteristics, regimen history and treatment outcomes. This pooled dataset will allow in-depth data analyses and will generate insights on HCV antiviral resistance not possible from individual studies. Through neutral, objective and independent, evidence-based approaches, the SHARED database can provide information to guide the clinical management of hepatitis C. An additional aim of the SHARED is to facilitate HCV resistance testing by providing HCV sequencing protocols, resistance interpretation software and relevant scientific literature. Knowledge in HCV antiviral resistance will be disseminated to the HCV communities through scientific presentations, publications, and a website.

C2. Objectives

There are 5 key objectives in the SHARED collaboration:

Objective 1: To collect HCV sequences, conduct resistance analysis of virologic failures, and define prevalence of known RASs and identify novel RASs..

Objective 2: To evaluate the impact of baseline polymorphisms in HCV on treatment outcomes.

Objective 3: To report the *in vitro* drug susceptibility of substitutions selected by approved direct-acting antivirals, and correlate biological cut-offs and clinical outcomes.

Objective 4: To describe the prevalence of genetic polymorphisms in HCV genotypes 4-6 and their distribution in different geographic regions.

Objective 5: To share technologies and know-how for resistance evaluation

D. METHODS

D1. Study Cohort

The SHARED database will consist of anonymized HCV sequences and their associated clinical and behavioural information from laboratories and clinics worldwide. Any individual identifiable data will be de-identified at the site of the respective contributing collaborators before depositing to the SHARED database. Data collection will occur through several data mergers over time. The first data merger will focus on information collected from patients who failed DAA therapy. As a control, data will also be gathered from a subset of DAA responders (SVR.). The first data merger will include sequences and data from approximately 7000 patients collected between 2011 – 2016 from laboratories in Canada.

This study will be conducted in accordance with the principles of the Declaration of Helsinki, and approval by local Ethics Committees will be obtained when deemed necessary.

- Key Inclusion Criteria: Any patient with acute or chronic HCV infection, naïve or experienced to PEG-IFN or DAA, age >18 years and with at least one HCV genotypic resistance test available on NS3 and/or NS5A and/or NS5B will be included in the study. Pre/post-OLT and decompensated cirrhosis, or HIV/HBV co-infections are not criteria for exclusion.

- Key Exclusion Criteria: Age <18 years. HCV-infected subjects with only HCV antibody without sequence data. Subjects with incomplete treatment information or outcome data will be excluded from Objectives 1 and 2 but can be included in Objective 4 for the epidemiology studies.

Since a majority of the studies during 2011-2016 were conducted in HCV GT1 infected individuals, and some studies were carried out in HCV GT2 – 4 infected individuals, the first data merger and the research will focus on these genotypes (Objectives 1–3). While sequence acquisition and investigation for HCV genotypes 4–6 will be conducted, the data analyses for these "rare" genotypes will likely take place during the second and subsequent data mergers (Objective 4).

D1.1 Cohort Profile

The data sets will contain some or all of the following information:
Demographic information collected at initiation of the HCV DAA therapy, including age, gender, ethnicity, BMI, country of birth, and clinical and behavioural factors (e.g. ongoing injecting drug use) associated with the development of RASs.

- Virology data: HCV viral load, genotype, HCV amino acid and/or nucleotide sequences
- Treatment data: treatment regimen and duration corresponding with when HCV sequences were generated, prior treatment history and response, and other relevant medications
- Host genetics data: e.g. IFNL3 (IL28B) polymorphisms
- Biochemistry testing data: alanine transaminase (ALT), aspartate aminotransferase (AST), bilirubin, hemoglobin, albumin, creatinine, international normalization ratio (INR), phosphate, urea, platelets, CD4 count, C-reactive protein, HBV and/or HIV co-infection status etc.
- Liver function data: liver fibrosis assessment, Child-Turcotte-Pugh class, MELD score, past orthotopic liver transplant, variceal bleeding, encephalopathy, ascites, hepatocellular carcinoma
- Extra-hepatic comorbidities: renal diseases, cardiovascular diseases, and cancers
- Treatment outcome data: SVR, non-response, viral breakthrough, relapse, drop-out, and reinfection

Transmission-related behaviour, including history of injecting drug use or opioid substitution therapy, and socio-economical information may be collected depending on the availability of data.

D2. Data Acquisition and Data Flow

The data sets will be collected under a medical or human research ethics committee approved protocol at each respective collaborator's site. Each contributing partner who has entered into the data sharing agreement will gather, computerize, and upload their anonymized data in a standardized format through a secured File Transfer Protocol (FTP) site. An automated quality check will be performed to ensure data integrity and consistency. Each patient sequence and the associated data will be assigned a unique coded identifier, merged, maintained and stored at the BC Centre for Excellence in HIV/AIDS (BC-CfE) with data protection security in place. The data sets will contain some or all of the information as listed in the cohort profile (D1.1 Cohort Profile).

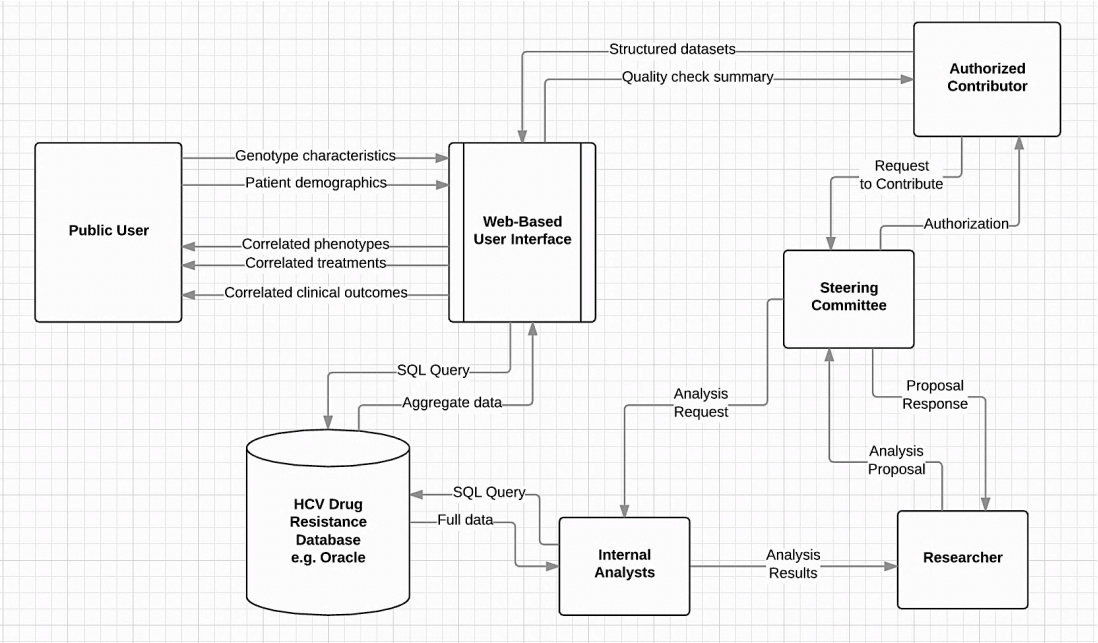
The primary objective of the SHARED is to provide simple, clear and "digested" interpretations of HCV antiviral resistance to patients and clinicians. It is envisioned that only aggregate analyzed data will be presented through the website, publications and public presentations. The reporting of individual anonymized sequences and their associated clinical information to the appropriate authorities due to requirements in the Public Health Act of British Columbia, Canada can be considered should the need arise; e.g. an outbreak of HCV resistant viruses where the identifying information allowing for such reporting is available. The output of the data analyses will be delivered through two channels: a web-based query system and investigator-driven research studies.

In the web-based query system (web database), virus genotypes, drug response, phenotypes, and clinical use cases will be modeled using an object-oriented analysis. System inputs and outputs will be presented using data-flow diagrams. Database users will define the parameters of their query using a user interface (UI). The search parameters will not be informative enough to identify the individual sequence or patient information. The database software will match the query and derive results. The database software will provide an UI for various functions, including genotype-treatment correlations, genotype-phenotype correlations, and genotype-clinical outcome correlations. Results will be displayed in aggregate to protect patient privacy. Available search results will include prevalence and characteristics of mutations, treatments, phenotypes, and clinical outcomes. The web-based queries will be accessible to public through personal usernames and password granted by the administrator from SHARED.

The SHARED collaboration provides an excellent opportunity to explore new hypotheses, plan research areas, and identify new treatment strategies and clinical management. In the investigator-driven research studies, individual-level records such as HCV sequence and its associated clinical information obtained as part of the SHARED will be available for research to the approved investigators following a research proposal submission. Data Transmission is encrypted and employs secure protocols for file transfer. It should be emphasized that all data received by SHARED are anonymized such that no personally identifiable information will/can be shared (patient level data are de-identified at the contributing collaborators' sites before depositing to SHARED). Most importantly, the data will not be available at the individual level, and privacy and security controls will be implemented in order to remediate any risks of re-identification. Plans for data analyses of the merged data set will be discussed with scientific experts and the

Steering Committee before conducting at BC-CfE or an appointed Institute. Similarly, consensus interpretations of the results will be adopted following discussions with scientific experts and the Steering Committee. The Steering Committee will be responsible for review and approval of concept sheets, data requests, and publications. All publications arising from the use of data from the Collaborative HCV Resistance Database shall comply with the ICMJE (International Committee of Medical Journal Editors) guidelines and the publication policies set up by the Collaboration.

An overview of the data flow is presented in Figure 1.



D3. Analytical Methods

To ensure concordance and reliability of data obtained from all participating centers, guidelines for analytical methodology will be developed and distributed. All centers are requested to adhere to guidelines to ensure high data quality.

Analytical methods will include (but are not limited to):

1. *HCV genotype*: HCV genotype and subtype determined by an approved commercially available genotyping assay, a validated research genotyping assay or direct HCV sequencing are acceptable. In the case of equivocal genotype determination, a phylogenetic analysis should be performed to confirm the genotypes. Procedures for phylogenetic analyses will be at the discretion of each collaborating center.
2. *HCV sequencing:* NS3, NS5A, and NS5B regions sequenced by either standard population sequencing (Sanger method) or by ultra-deep sequencing are acceptable. Consensus sequence data can be provided in FASTQ or FASTA format with quality metrics. A cut-off of 15% will be used for variant mixture determinations. Additional lower sequencing cut-offs will be evaluated for

sequences generated using NGS to determine the impact of minority RASs on treatment outcomes in Objectives 1 and 2.

3. *HCV viral load*: HCV-RNA should be quantified using an approved commercial assay, such as COBAS Ampliprep/COBAS TaqMan HCV quantitative test v2.0 (Roche Diagnostics, Mannheim, Germany), the Abbott RealTime HCV assay (Abbott Laboratories, Illinois, U.S.A.). The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) for these assays are 12-15 IU/mL and 12-25 IU/ml, respectively. Locally approved or home-grown validated RNA assays can be used at the discretion of the each collaborating center and approval of the SHARED steering committee.
4. *Resistance Interpretation*: The impact of a viral substitution on antiviral resistance is defined by replicon EC_{50} FC and if the substitution is associated with virologic failure in patients. The values of EC_{50} *or* EC_{50} FC will be obtained through a literature search (see Objective 4).
5. Clinical measurements: Standard biochemical assays used by the respective centers are acceptable.

Statistical methods will be used to describe the prevalence and characteristics of substitutions, treatments, phenotypes, and clinical outcomes. Qualitative variables will be presented as frequencies and proportions. For quantitative variables means (standard deviation), medians (25th; 75th percentiles) and ranges (minimum-maximum) will be given. Percentages will be compared using the Chi-Square or Fisher exact tests, medians will be compared using the Wilcoxon test and means will be compared using Student's t-test. To analyze factors associated with binary outcomes (e.g. SVR) multivariable logistic regression will be used. Survival curves for cohort retention will be estimated using the Kaplan-Meier method. Cox proportional hazard models may be used to evaluate time to event analyses associated with treatment outcomes. Missing values will be considered to be missing at random.

D4. Data Analysis by Study Objectives:

Objective 1: Sequence collection and resistance analysis of virologic failures.
Led by Drs. Federico Garcia, Francesca Ceccherini-Silberstein

Hypothesis: Patients who fail to achieve SVR after DAA therapy are likely to harbor RASs. RASs detected at HCV RNA recurrence can be present at low levels before treatment, and enriched during treatment. Similar RASs or RAS patterns are expected in patients with different disease characteristics. The persistence of RASs varies among different target genes.

A prospective cohort of HCV-infected subjects who failed DAA therapy according to the inclusion and exclusion criteria will be established. Anonymized HCV sequences and the associated demographic and clinical data from this cohort will be collected from academic or hospital laboratories, resistance testing facilities, clinical trials, HCV network or consortium, or databases such as VIRONET-C, GEHEP-004 and HepCare.

This work-stream will thus contain a large number of DAA-treated patients from diverse clinical settings. The data analyses will allow a comprehensive characterization and surveillance of HCV resistance in patients who failed DAA therapies.

Resistance profiles for each approved DAA and drug class stratified by HCV genotypes and subtypes will be assessed to:

1. Identify specific RASs or RAS patterns selected by each approved DAA or drug class;
2. Compare the prevalence of mutations in NS3, NS5A, and NS5B between DAA-treated and untreated patient populations;
3. Identify treatment-emergent RASs versus persistent baseline RASs in virologic failures with paired baseline and virological failure sequences;
4. Correlate RASs and RAS patterns with demographic and clinical characteristics;
5. Estimate the persistence of RASs over time;
6. Evaluate the impact of resistance guided therapy on SVR;
7. Compare the frequency of RASs detected by Sanger population sequencing versus ultra deep-sequencing with different sequencing cut-offs.

Web-based queries and outputs:

- Treatment profiles: mutation frequencies at each position of the target gene for the drug(s) specified. Results are displayed according to genotype and subtype
- Mutation profiles: prevalence of mutations at the position of interest within the target gene in untreated persons, and in persons receiving the specified drugs. Statistical difference correlates the likelihood of mutations in treated vs. untreated persons
- Detailed treatment queries: major and minor substitutions observed in individual patient isolates according to the specified drug class and drugs (individual's HCV sequences will not be displayed)
- Detailed mutation queries: major and minor mutations observed in individual patient isolates at the positions of interest (individual's HCV sequences will not be displayed)
- Longitudinal resistance profiles: major and minor mutations observed in longitudinal patient isolates according to the specified drug history
- Summary of substitutions: prevalence of clinically relevant substitutions in drug-treated and untreated population according to genotype, drug(s) used, demographics, clinical characteristics, and treatment history

Sample Size and Statistical Plan:
Approximately 2000 HCV sequences from NS3, NS5A and NS5B genes will be collected during the first data merger and used for the resistance analyses. Statistical differences in the demographics, clinical characteristics and resistance profiles between treated and untreated subjects will be assessed by using Chi Square test or Fisher's exact test (for categorical variables) and Mann-Whitney test (for continuous variables) or Logistic Regression Models, as appropriate.

Objective 2: Impact of baseline polymorphisms on treatment outcomes

Led by Dr. Valeria Cento and Anita Howe

Hypothesis: Patients who harbor HCV variants with reduced drug susceptibility are likely to respond poorly to DAA treatments. The impact of baseline polymorphisms on treatment outcomes varies depending on the type of substitutions, patient, clinical and behavioural characteristics (e.g. ongoing injecting drug use), and the drug class.

This study will include all patients with baseline HCV sequence for whom the final treatment outcome is available.

The prevalence of specific polymorphisms in HCV NS3, NS5A and NS5B genes, present prior to therapy, in patients achieving SVR will be compared with those in patients who had virologic failure. Additionally, different sequencing thresholds will be used in samples with NGS sequencing data to evaluate the impact of minority RASs. Polymorphisms beyond the target gene regions may be explored. The analyses will be carried out accounting for the DAA used, and HCV genotype/subtype.

Subgroup analyses will be conducted to understand how host and viral factors might affect the impact of baseline HCV polymorphisms on treatment outcomes. Relevant host factors and clinical characteristics will include age, gender, ethnicity, recent injecting drug use, cirrhosis, prior treatment history and response, IL28B, HIV or HBV-co-infections, and possible route of transmission. Viral factors will include viral load, genotype/subtype, sequencing cut-off, the number of RAS, RAS combination, and the phenotypic resistance profile of RAVs.

Web-based queries and outputs:

- Substitution profiles: the prevalence of substitution at the position of interest within the target gene in untreated persons, and in persons receiving the specified drugs. Statistical difference correlates the likelihood of mutations in treated vs. untreated persons
- Clinical profiles: the prevalence of key clinically relevant mutations in patients with specified clinical characteristics
- Distribution profiles: the prevalence and the geographic distribution of mutations at the position of interest within the target gene

Sample Size and Statistical Plan:
The first merged dataset will contain approximately 3000 baseline sequences from subjects who meet the specified inclusion criteria. Approximately equal numbers of sequences will be derived from subjects who had SVR, and from subjects who experienced virologic failure. Odds ratio and/or Fisher exact test will be used to determine the association of the baseline polymorphisms with treatment failure. Cox regression analysis, uni- and multivariable, will be used to estimate the predictive impact on the virological response of RASs and other clinical and virological covariates. A Receiver-Operating Characteristic (ROC) analysis will be conducted to determine the optimal sequence cut-offs to predict clinical outcomes.

Objective 3: *Phenotypic Resistance*
Led by Dr. Johan Lennerstrand and Charles Boucher

Hypothesis: In vitro EC_{50}, to some extent, predicts drug susceptibility of RASs in vivo. Phenotypic cut-offs, as determine by the EC_{50} fold-change (FC), can provide information about the likelihood of the virus responding to treatment with a particular drug.

In this study, EC_{50} or EC_{50} FC data for RASs in HCV NS3, NS5A and NS5B from GT1–6 will be collected through literature search in Medline, product labels, briefing documents, advisory reviews, and reports from international congresses. Investigators are also invited to contribute unpublished data to the Collaborative HCV Resistance Database.

EC_{50} data generated from stable replicons or transient HCV replicons with a reporter gene will be included in the analyses, provided that the EC_{50}s for the wild-type and mutant replicons were generated from the same system with the same genetic background (i.e. same strain and GT/subtype). The phenotypic resistance of a drug-specific RAS, defined as fold-decreased susceptibility (EC_{50} FC) of a replicon containing a mutation compared to the wild-type, will be reported for each approved drug in each genotype. The threshold of phenotypic resistance that distinguishes between responders and non-responders for the RASs of interest will be estimated using a ROC analysis for each drug in each genotype.

Web-based queries and outputs:

- A comprehensive summary of *in vitro* EC_{50} and EC_{50} FC for each approved drug against RASs that are selected *in vitro* and *in vivo*
- The EC_{50} FC and the frequency of RASs observed in untreated and treated patient population.
- The resistance level of RASs e.g. low, intermediate, and high levels of resistance based on their *in vitro* EC_{50} FC, the frequencies and the modality of their selection associated with virologic failure
- Literature reference(s) for each reporting

Sample Size and Statistical Plan:
During the first data merger, approximately 300, 150, and <50 records will be collected for the NS5A RASs in GT1a/1b, GT3, GT2/5/6, respectively. The information of NS3 RASs will be limited to GT1a and 1b; about 150 records will be reported for each subtype. About 80 NS5B RASs specific to dasabuvir in GT1a and GT1b, and <50 NS5B RASs specific to sofosbuvir in GT1-6 combined will be recorded. Odds ratio and/or Fisher exact test will be used to determine the association of the RASs with treatment failure. A ROC analysis will be conducted to estimate the threshold of phenotypic resistance for predicting clinical outcomes.

Objective 4: Resistance-associated variants in the "rare" HCV genotypes (GT 4 – 6)
Led by Drs. Tanya Applegate and Anita Howe

Hypothesis: Drug resistant variants in the "rare" HCV genotypes (GT 4 -6) will lead to poor treatment outcomes similar to GT1 strains. The prevalence and distribution of these resistant viruses vary in different geographic locations.

In the first phase of this project, sequences from HCV genotypes frequently circulating in Asia, Latin America and Africa will be acquired. Similar to the sequence acquisition for GT 1–3, sequences will be collected from prospective cohorts, clinical trials, academic laboratories, resistance testing facilities, and national or regional databases. Demographic information, virology data, treatment records, clinical characteristics, treatment outcomes, and host genetic information associated with the sequences will be collected, when available (see D1.1 Cohort Profile). Similarly, *in vitro* EC_{50} and EC_{50} FC of RASs will also be acquired. It is anticipated that many of the countries in these regions might not have a well-established sequencing facility and consequently, the data mentioned above might be limited. A separate work stream (Objective 5) will be set up to facilitate access to sequencing technology and knowledge translation.

The second phase of this project will involve phylogenetic analysis, and genotype/subtype assignment of the collected sequences using the criteria proposed by Smith et al. (*43*) Sequences from the same genotype will be aligned to interrogate the frequency of polymorphisms. The prevalence and the geographic distribution of polymorphisms in treated and untreated patient populations will be reported.

The third phase of the project will address several key research questions including:

1. The prevalence and distribution of naturally-resistant HCV strains to NS3-protease, NS5A or NS5B inhibitors
2. Resistance profile in virologic failures treated with DAA therapies (see Objective 1)
3. The impact of baseline resistance on treatment outcomes (see Objective 2)
4. Phenotypic resistance of RASs (see Objective 3)

Sample Size and Statistical Plan:
Not available

Objective 5. Technology and Knowledge Sharing
Led by Drs. Richard Harrigan and Tanya Applegate

Hypothesis: Availability of sequencing methods and analysis software will facilitate access and enhance uptake of resistance testing. Standardized approaches and guidelines will ensure concordance and reliability of data from all participating centers. Knowledge users are likely to use the research results to make informed decisions about treatment strategies and health policies.

The purpose of this work-stream is to share know-how and research tools for the study of HCV resistance. The content of this work-stream will be delivered primarily through the SHARED website, where information and methodologies concerning HCV resistance can be retrieved readily through a user interface. This work stream will cover 3 key areas:

1. HCV assays - sequencing, genotyping and viral load
2. Data analysis software and interpretation systems
3. Literature on HCV clinical studies and resistance

5.1 HCV assays – sequencing, genotyping and viral load

5.1.1 Sequencing

A systematic review will be conducted to evaluate available HCV sequencing methods for RAS detection. A public library of published sequencing primers for HCV GTs 1–6 will be developed. Investigators will also be invited to contribute unpublished methods. The PCR primer library will contain primer sequences and locations, sequences, amplicon length, HCV gene coverage, genotype specificity and references. Expert guidance on the selection of the most appropriate methods will also be provided. Sequencing methods, including Sanger population sequencing and NGS that have been validated using clinical samples will be included. Assay performance characteristics, including the limit of detection, key RAS coverage in the protein coding regions of NS3, NS5A and NS5B, and robustness, will also be reported, when available.

5.1.2. Genotyping

This section will provide a summary of the assay performance characteristics of key commercially available genotyping assays. A list of web-links to the respective sites will be included.

An open-source research tool, using a phylogenetic approach and HCV sequences to determine HCV genotype, will be developed. Users will be required to upload a consensus HCV sequence in a FASTA format through a user interface to obtain the genotype/subtype assignment.

5.1.3 Viral Load

This section will provide a summary of the assay performance characteristics, including the lower limit of detection (LLOD) and Lower Limit of Quantification (LLOQ), of standard commercially available HCV RNA assays. A list of web-links to the respective sites will be included.

5.2 Data analysis software and interpretation systems

Two software applications, RECall and MiCall, with a user-friendly interface will be developed to analyze HCV sequences and interpret antiviral resistance. RECall is a fully automated, custom sequence analysis web application originally developed for HIV drug resistance genotyping. It is currently used by WHO for HIV resistance interpretation.(*44*) Sequencing trace files (i.e. raw ABI chromatograms) generated from population sequencing can be directly input into RECall to produce a consensus sequence aligned to a user-supplied reference sequence (e.g. HCV GT1a_H77) with minimal hands-on time. Mutations in amino acids and nucleotides, peak heights for the associated nucleotide

mixtures, and the quality score for each sequence fragment are reported. The current version, available at http://pssm.cfenet.ubc.ca, will be further developed to incorporate antiviral resistance interpretations at key RAS positions for HCV.

MiCall is a software program developed by BC-CfE to process the raw short reads generated by the Illumina MiSeq next-generation sequencing platform. MiCall comprises a "backend" that automates MiSeq data (FASTQ) processing and analysis, and a "frontend" that allows users to evaluate result summaries and approve or disapprove samples based on various quality indicators. The result summaries include run-level quality statistics (cluster density, % clusters passing filters, %bases >Q30, number of cycles/tiles with high error rates), final consensus nucleotide sequences, amino acid frequencies, and viral genotypes.

5.3 Literature on HCV clinical studies and resistance

This sub-section will provide a list of references with downloadable PDFs on pivotal clinical trials for the approved drugs, and investigational drugs that are in Phase 2/3 development. Special emphasis will be placed on literature that contains resistance information. Publications on *in vitro* resistance selection and characterization will also be included.

E. TRAINING AND TUTORIAL ACTIVITIES

The project involves training of biologists, biotechnologists and laboratory technicians in the context of HCV viral sequencing. The training will take place through sharing of protocols, cross-validation of the results, and in-person training of researchers within the network of participants. The laboratory staff will also be trained in procedures for the acquisition, control and storage of biological samples, through the organization of *ad hoc* seminars. Specific training for the interpretation of tests and results obtained during the project will be possible through periodical meetings.

The success of this project will rely solely on collaboration among the network participants, as there is no formal sponsorship or funding for organization of events. The Collaboration will serve as a channel where requests for training and collaboration can be communicated among participating members.

F. RISK ANALYSIS, POSSIBLE PROBLEMS, AND SOLUTIONS

The potential critical difficulties of this project involve data collection and development of a standardized methodology for HCV resistance testing and resistance interpretation. However, the extensive, global network that this project aims to establish will allow access to a large number of samples, along with clinical and virological information. The collaboration with the already-established national HCV-networks such as the Italian VIRONET-C, the HCV TARGET, the BC Centre for Excellence in HIV/AIDS, the Spanish GEHEP-004 and the Kirby Institute in Australia among other participants will provide an enriched source of standardized methodologies for HCV sequencing and experience in HCV resistance interpretation. Overall, the expertise of the present group

places us in a pre-eminent position to generate innovative data that fulfill the aims of this project.

The nature of SHARED which involves an exchange of patient data, patients' personal information can move quickly around the globe if there is no secure data protection policy in place. While every participant in SHARED will endeavour to protect patient privacy, the risk for data protection remains. The table below indicates some risks involved in this collaboration and the potential mitigation strategies.

	Risk	**Mitigation Strategy**	**Likelihood**	**Impact**
1.	Employees of the Collaborating Parties could access personal information and use or disclose it for personal purposes	Restricted, role-based access, auditing of data access and use, confidentiality agreements, professional ethical duties, and contractual terms	Low	High
2.	Patient's personal information is compromised when transferred to the BC-CfE for linkage	Patient's personal information will be de-identified at the respective contributing investigators' sites before transmission. Transmission is encrypted and employs secure protocols for file transfer	Low	High
3.	Collaborating Parties' processes, technology and policies and practices are not within the control of any one Collaborating Party	Collaborating Parties' are legally responsible for managing these risks; Privacy Impact Assessment (PIA) process identifies risk and mitigation strategies to be implemented; Terms of Agreement (ToA) imposes contractual duties on the Collaborating Parties that are negotiated between them	Low	High
4.	Re-identification of de-identified data (ie, if small cell size becomes an issue in particular the information is associated with geographic information)	If there are fewer than five individuals in any analytic or reporting cell posing any risk of identification, BC-CfE will re-categorize the variable or collapse cells to ensure that these individuals are not identifiable in any reported results. Geographic information will be restricted at the regional level such that an individual cannot be re-identified.	Medium	High
5.	Data disclosed to Collaborating Parties used for purpose unrelated to the Project	Agreements, including the ToA, between the Collaborating Parties; staff agreements such as Confidentiality Acknowledgements or Pledges, contracts and education	Low	Medium

Finally, there is a potential risk with regards to the sustainability of the project, which depends on the availability of financial support. For the first data merger and analyses,

each participating center is contributing data and resources to seed this project. It is anticipated we will initiate discussions with potential funders and collaborating partners including Bill and Melinda Gate's Foundation, FIND/UNITAID, National Institute of Health (NIH), Canadian Institutes of Health Research (CIHR), EASL Initiative grant, pharmaceutical industries and local funding agencies. Joint grant applications will be submitted to multiple agencies to request funding to support this collaboration.

G. SIGNIFICANCE AND INNOVATION

Through a joint effort of clinicians, virologists and researchers, this project aims to widen the knowledge on clinically meaningful HCV RASs, enhance access to resistance testing and guide clinical management of HCV infection in the context of antiviral resistance.

The identification of pre-treatment predictors of virologic outcome to DAA therapies, including the identification of the most clinically significant RASs, will enable clinicians to choose the best treatment strategies with the highest success rates.

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