# HALT-C Ancillary Study PROPOSAL

**Part I** (1 page)

**Proposal Name: Hepatitis C Virus Quasispecies in the Resistance to Antiviral Therapy**

Proposal PI: Xiaofeng Fan, MD, PhD. Investigator (Saint Louis University)

**Co-Investigators:** Adrian M. Di Bisceglie, MD. (Saint Louis University)
John E. Tavis, PhD (Saint Louis University)

**HALT-C PI:** Adrian M. Di Bisceglie, MD (Saint Louis University)

**Funding Agency and Review Body** (e.g., NIDDK; my university/GAC): NIDDK

I agree to follow HALT-C Policies and Procedures when conducting this study. I acknowledge that the data obtained from this study will belong to the NIH and will be placed in the HALT-C database for use by other investigators. I understand that I cannot begin experiments using HALT-C specimens/data until I receive approval from the HALT-C Steering Committee and funding from the Scientific Review Body for my proposal. I also understand that the data analysis for this proposal will be performed by NERI (unless otherwise approved by the HALT-C study) and that Protocols approved by the HALT-C Steering Committee will be placed on the HALT-C Restricted Website.

| | |
|---|---|
| Proposal Principal Investigator | Date |
| HALT-C Principal Investigator Date | Date |

**Protocol Part II** (4 page limit, single space)

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**1. Specific Aims**

Hepatitis C virus (HCV) infection is one of the major concerns in public health. Over 2.7 million Americans are chronically infected with HCV, which results in ~10,000 deaths each year and is a leading cause for liver transplantation. Currently, optimal antiviral therapy with pegylated interferon-alpha plus ribavirin, cures ~50% of patients infected with HCV genotype 1 and ~80% of patients infected with HCV genotypes 2 and 3. It is not known why about half of patients infected with HCV genotype 1 have no response to antiviral therapy and why there is such a remarkable difference in response between HCV genotypes. Among the difficulties regarding our understanding on these issues, one is related to the nature of current therapeutic agents. Both interferon and ribavirin have long been known for their broad-spectrum antiviral activity by creating a non-specific antiviral status rather than the direct interaction with viruses. As a result, no explicit targets on HCV genome have been documented. Accordingly, studies on drug resistance with these agents resulted in limited understanding and generated controversial data through conventional approaches that frequently focus on short domains of the HCV rather than the entire viral genome. Furthermore, the data interpretation from most studies, if not all, is weakened by the simultaneous involvement of many other factors that are independently associated with therapeutic results. By using well-characterized serum samples from the Hepatitis C Antiviral Long-term Treatment against Cirrhosis trial (HALT-C), we propose a viral sequencing project to study viral mechanisms of resistance using a novel long RT-PCR and cloning technology. The large study population of HALT-C trial allows the design of well-controlled experiments that minimize the role of host factors related to therapeutic responses. Viral mechanisms of resistance to antiviral therapy will be exhaustively examined at multiple levels with a focus on viral quasispecies structures.

***HYPOTHESIS:*** ***The main hypothesis to be tested in these proposed studies is that HCV resistance to antiviral therapy is associated with region-dependent mutations of viral quasispecies at either single variants or the population level.***

**Specific Aim 1: To explore genetic signatures at both HCV isolate and quasispecies levels in null responders infected with HCV genotype 1a.** HCV genotype 1a is a predominant subtype in the United States. About 50% of patients infected with HCV genotype 1a have no response to current antiviral therapy. Such remarkable differences are hard to explain solely by host factors. Viral mechanisms, acting on either isolate- or quasispecies level, must play an important role. Thus, the full-length HCV quasispecies profiles as well as consensus sequences derived from quasispecies population at the baseline will be generated from HCV genotype 1a patients with null and sustained virological responses (SVR). Comprehensive comparative analyses at multiple levels will be performed between these two groups to identify potential genetic signatures that are associated with the treatment resistance.

**Specific Aim 2: To demonstrate if there are distinct quasispecies structures of HCV genotype 2 in terms of the high response rate to the antiviral therapy.** Among many studies regarding HCV antiviral therapy, there are two clinical observations that are well documented in past decades. First, patients infected with HCV genotype 2 have a much higher response rate (~80%) comparing to HCV genotype 1. Second, acute HCV infection, regardless of HCV genotype, can be cured in ~80% patients. Taken together, these observations indicate that HCV is not intrinsically resistant to antiviral therapy. Therapeutic differences must be attributed to viral factors associated with chronic HCV infection. In this setting, the establishment of a well-adapted, dynamic HCV quasispecies population is perhaps the most salient feature during chronic infection. We thus hypothesize that quasispecies structures are responsible for therapeutic difference between HCV genotypes 1 and 2. To test this hypothesis, the full-length HCV quasispecies profiles will be generated from twenty sustained virological responders infected with HCV genotype 2a, followed by comparative analyses with those derived from HCV genotype 1a as described in Specific Aim 1.

**Specific Aim 3: To characterize mutational patterns associated with HCV re-emergence in patients with relapse after initial response to antiviral therapy.** The relapse, viral reemergence after the drug withdrawal, is a major concern during antiviral therapy. Since there is no evidence for its integration into cellular genomes, HCV must continue to replicate for survival albeit at a very low level, below the detection

limit of current RT-PCR technique, which forms a so-called population bottleneck in terms of evolutionary biology. The continuing accumulation of deleterious mutations under population bottleneck will eventually drive the virus to extinction, known as Muller's Ratchet as confirmed by experimental models. Unusual mutations in certain domains and recombination are two major behaviors for the virus to survive the population bottleneck. There are no data about how HCV responds to such "*in vivo*" population bottlenecks created by antiviral therapy. This issue will be addressed through a sequential comparative analysis of full-length HCV quasispecies profiles derived at baseline and the time of first re-appearance of HCV in patients with relapse response.

*Clinical strength (samples from HALT-C trial), technical innovation and power (novel long RT-PCR and cloning) and the application of evolutionary biology are three essential components of this application. Their combination will allow us to investigate novel hypotheses regarding to viral factors that contribute to treatment resistance of chronic HCV infection.*

## 2. Background/rationale

*2.1. Hepatitis C Virus.*

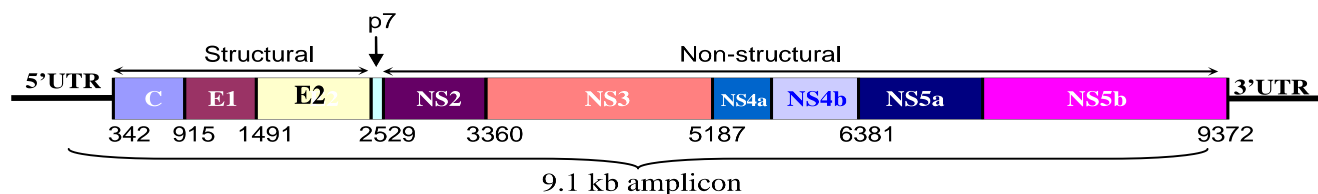


**Figure 1. Organization of the HCV genome.** The 5'UTR and 3'UTR are shown as lines. Between them is an open reading frame encoding a single polyprotein processed into structural or non-structural proteins as shown in boxes. Nucleotide numbering is according to HCV H77 strain, GenBank accession number AF009606. The target domain for the long RT-PCR, about 9.1 kb, is also indicated.

*2.2. HCV Genetic Variability.* The HCV genome is highly variable. Based on the phylogenetic analysis of nucleotide sequences, HCV is divided into at least six major genotypes (20-30% sequence difference) and more than 50 subtypes (10-20% sequence difference) (31). Moreover, even within an individual infected with a single HCV subtype, HCV circulates as a group of different but genetically closely related variants, referred to as viral quasispecies (less than 10% sequence difference), a characteristic shared by most of RNA viruses (2, 8). The molecular basis for HCV quasispecies nature is the high viral turn-over rate (about $10^{12}$ virions per day (23) and the high error rate of its RNA-dependent RNA polymerase encoded by HCV NS5B, which lacks proof-reading repair activity (15). Although the variability has been well documented across the entire HCV genome (reviewed in 20, 30), the most variable regions are located on envelope domains. In particular, the 5' end of the second envelope sequence, an 81 bp domain, has been shown to be extremely variable, named hypervariable region 1 (HVR1) (14).

Additionally, there is increasing evidence that HCV population in infected individuals contains defective genomes missing partial viral sequences (24, 34). Using long RT-PCT (LRP) technique, we found that HVR1 profiles from 9.1 kb amplicon (near full-length HCV genome) were different from that derived from 1.38 kb fragment, indicating the possibility that some HVR1 variants came from defective HCV genome (13).

*2.3. Quasispecies, quasispecies theory and factors shaping quasispecies distribution and evolution.* The term "quasispecies" is now over-used to describe the intra-patient variability of a given microbe, even including bacteria (16), which is to some extent away from its original definition by Dr. Eigen (1). Aiming to describe error-prone replication of simple RNA or RNA-like molecules, the quasispecies is defined as equilibrium mutation distributions of infinite size, centered around one or several master sequences (9, 10). Quasispecies theory has been applied to virology since mid-1980s, exclusively for the RNA viruses that circulate as a heterogeneous but not clonal population in infected individuals (3). Although there are many overlaps with classical population genetics, one of the unique assumptions in quasispecies theory is that all

viral variants, therefore the quasispecies, evolve as a unit. Consistent with this hypothesis, RNA viruses respond to external pressure, such as antiviral therapy, at the quasispecies (unit) level rather than as individual viral variants. The fate of single viral variants is not only dependent on their intrinsic biological characteristics but is also affected by other variants in the population. Thus all viral variants form a cooperative network, named quasispecies structure (4, 5). In spite of some conceptual debates (2), quasispecies theory has been demonstrated on experimental models with RNA viruses, and well developed by continuing integration from experimental and theoretic evidence, such as clonal interference (21) and molecular memory (27).

Both external and internal forces drive quasispecies distribution and evolution. The former has been well documented in clinical studies on viral quasispecies, such as antiviral immunity and drug therapy. The latter, mostly studied under experimental settings, comes from viral intrinsic nature to climb fitness peaks on a rugged landscape (6) and plays a role particularly on those putative neutral (mostly synonymous) sites along with entire viral genomes (17). This situation is largely ignored in most clinical quasispecies studies that frequently focus on small viral regions, especially on those domains known to be sensitive to immune responses. HCV HVR1 is a good example because both B-cell and T-cell epitopes have been identified within this 81-bp region (25, 36). The full-length viral quasispecies analysis represents the optimal approach to obtain a full picture of viral quasispecies.

*2.4. Technical Considerations in HCV genetic studies.* Comparative genetic analysis in well-controlled clinical samples is a classical approach to locate sites/domains on viral genome that are associated with drug resistance, as seen with HIV and hepatitis B virus. However, unlike protease/polymerase inhibitors or nucleotide analogues, both interferon-$\alpha$ and ribavirin have no explicit target. Thus any attempts to identify viral determinants should be performed at the full-length HCV genome level. Unfortunately, there are few studies to be conducted at the full-length HCV genome level (7, 11). More comprehensive analyses based on full-length HCV quasispecies profiles have not been reported.

The recovery of viral genome using RT-PCR technique is biased by many factors, such as errors introduced by DNA polymerase, the amplification bias due to primer choices as we demonstrated previously (12) and the existence of defective HCV genomes that complicate sequence interpretation especially when defective HCV genomes dominate viral population. Additionally, even in studies with full-length HCV sequences, these sequences are assembled from multiple PCR products covering the entire HCV genome. Thus potential linked mutations (covariance) in authentic HCV genome, as indicated by our recent study (35), may not be maintained. Taken together, technical factors may be important contributors to controversial reports in exploring viral determinants responsible for differential results of HCV antiviral therapy.

*2.5. Long RT-PCR technique (LRP).* In spite of several previous reports for the amplification of near full-length HCV genome (18, 28, 32, 33), experiments with these protocols are not reproducible. We have investigated every step for the LRP procedure and developed a robust protocol for the efficient amplification and cloning of near full-length HCV genome from clinical samples (13). Our LRP protocol has the following characteristics. First, it is robust and highly reproducible with a 96% amplification rate among 53 serum samples tested so far; Second, PCR-mediated recombination is not detected; Third, genetic diversity is preserved; Finally, a newly developed vector, pClone, allows efficient cloning of large amplicons. This technology has been validated by successfully separating near full-length HCV quasispecies variants from a complex population (35).

**3. Relations to aims of HALT-C study**

The HALT-C is one of the largest clinical trials, which aims to determine the effect of long-term peginterefron therapy on clinical and histologic progression in patients with chronic HCV infection (18). All patients recruited into this trial had failed previous antiviral therapy, including interferon $\alpha$ (18). The HALT-C trail includes numerous ancillary studies, including the research to identify factors responsible for differential therapeutic responses from either host or viral side, such as viral quasispecies diversity and putative neutralizing antibodies (22, 26). Thus our proposal completely matches up with aims of the HALT-C trail.

Comparative viral genome analysis, a major research tool in our proposal, requires well-controlled groups to avoid systematic bias. In this setting, HALT-C trial provides a great opportunity to allow the selection of appropriate groups of patients for our project.

**4. Study design**

*4.1. Outline:*

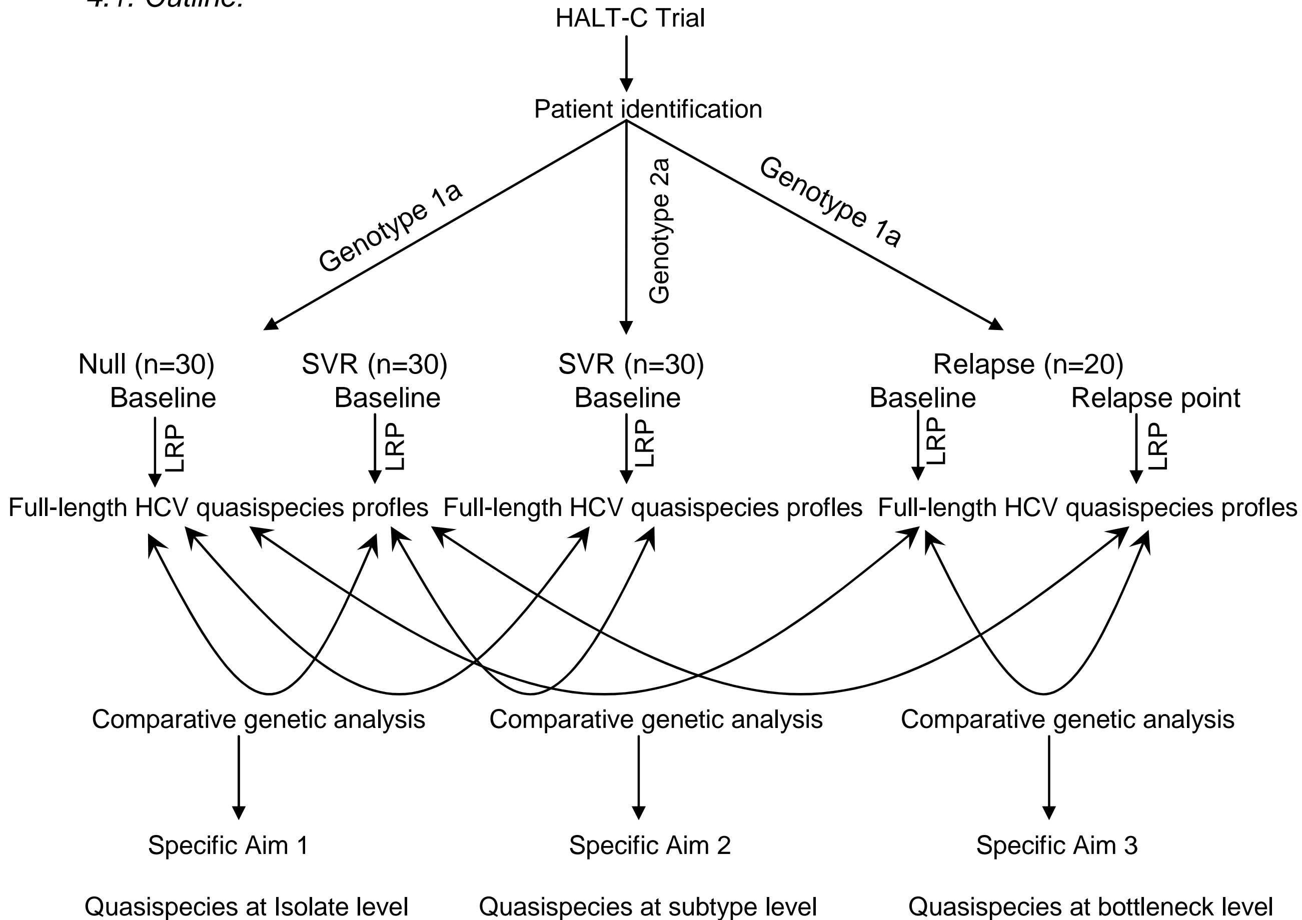


**Figure 2.** The outline of study design.

*4.2. Samples from HALT-C trial.* We will utilize a total of 120 serum samples collected from 100 patients in HALT-C clinical trial, which provides a large repository for sample selection (Table 1). All samples used in this proposal should be matched between groups with regard to demographic factors that are associated with final therapeutic results, as reported from HALT-C trial (29).

| Patient group | Response pattern | Number | Genotype | Time points | |
|---|---|---|---|---|---|
| 1 | Null | 30 | 1a | Baseline | |
| 2 | SVR | 30 | 1a | Baseline | |
| 3 | SVR | 20 | 2a | Baseline | |
| 4 | Relapse | 20 | 1a | Baseline | The first re-appearance |
| Total | | 100 | | | |

**Table 1. Summary for serum samples requested from HALT-C.**

*4.3. LRP and cloning:* Experimental protocols are detailed in our recent publications (13, 35).

*4.4. Comparative genetic analysis.*

4.4.1. Does genetic complexity or diversity correlate with response patterns of antiviral therapy?

4.4.2. Is there covariance within or among HCV functional genes?
4.4.3. Does recombination occur in quasispecies variants responsible for the relapse?
4.4.4. Are there different amino acid hydrophobicity patterns related to the antiviral therapy?
4.4.5. Are there specific mutation patterns associated with HCV relapse?
4.4.6. Does HCV evolve toward the resistance of current antiviral therapy?

*4.5. The establishment of a specific HCV clone bank.*

**5. Data from HALT-C:** NERI is now preparing a subcontract from this application for sample management and data processing, including viral loads, genotypes and other clinical information.

**6. Anticipated results:** We do not anticipate any difficulties in completing experiments in this proposal. Core techniques are well established in the lab and the investigators are skillful in all types of viral genetic analyses. Our hypothesis will be examined using the most comprehensive genetic analysis. A specific clone bank allows future *in vitro* tests when HCV cell culture becomes mature. These clones are valuable for estimating antiviral activity of new drugs since combinational antiviral therapy is a current consensus.

**7. Statistical support:** Genetic analysis will be performed by using multiple programs, including Clustal W, MEGA, PAUP, PAML, GCG and BioEdit etc as seen in our previous publications. Values of genetic parameters from these analyses will be tested for statistical significance using paired Student's t test or Mann-Whitney Rank Sum Test. Categorical data from cross analyses will be tested for statistical significance using the $^2$ test with Yate's correction or Fisher's exact test. All statistical analyses will be done using SPSS (version 11.0), SigmaStat (version 3.5) and SigmaPlot (version 10).

**8. Financial issues:** We will submit this proposal to NIH in response to PAR 07-024: Ancillary Studies to Major Ongoing NIDDK and NHLBI Clinical Research Studies (R01). The deadline for next cycle is June 5, 2007.

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**Protocol Part III: Sample Requirements. 30 patients with null responses (genotype 1a)**

| Visit | Liver# patients, mm* | Blood# patients, ml | DNA# patients, ug | Liver Biopsy Slides# patients, slides/patient | Other (describe)# pts, amount |
|---|---|---|---|---|---|
| Screen 1 | | | | | |
| Screen 2 | | | | | |
| Baseline | | 0.5 ml x 30 pts | | | |
| Lead in Week 4 | | | | | |
| Week 8 | | | | | |
| Week 12 | | | | | |
| W16 | | | | | |
| Week 20 | | | | | |
| Week 24 | | | | | |
| Randomized Month 9 | | | | | |
| Month 12 | | | | | |
| Month 15 | | | | | |
| Month 18 | | | | | |
| Month 21 | | | | | |
| Month 24 | | | | | |
| Month 27 | | | | | |
| Month 30 | | | | | |
| Month 33 | | | | | |
| Month 36 | | | | | |
| Month 39 | | | | | |
| Month 42 | | | | | |
| Month 45 | | | | | |
| Month 48 | | | | | |
| Post-treatment | | | | | |
| Responders W30 | | | | | |
| W36 | | | | | |
| W42 | | | | | |
| W48 | | | | | |
| W60 | | | | | |
| W72 | | | | | |

**We need all demographic data (age, sex and race) and viral load.**

**Samples are restricted to HCV genotype 1a.**

**This is for the Specific Aim 1.**

**Protocol Part III: Sample Requirements. 30 patients with SVR (genotype 1a)**

| Visit | Liver# patients, mm* | Blood# patients, ml | DNA# patients, ug | Liver Biopsy Slides# patients, slides/patient | Other (describe)# pts, amount |
|---|---|---|---|---|---|
| Screen 1 | | | | | |
| Screen 2 | | | | | |
| Baseline | | 0.5 ml x 30 pts | | | |
| Lead in Week 4 | | | | | |
| Week 8 | | | | | |
| Week 12 | | | | | |
| W16 | | | | | |
| Week 20 | | | | | |
| Week 24 | | | | | |
| Randomized Month 9 | | | | | |
| Month 12 | | | | | |
| Month 15 | | | | | |
| Month 18 | | | | | |
| Month 21 | | | | | |
| Month 24 | | | | | |
| Month 27 | | | | | |
| Month 30 | | | | | |
| Month 33 | | | | | |
| Month 36 | | | | | |
| Month 39 | | | | | |
| Month 42 | | | | | |
| Month 45 | | | | | |
| Month 48 | | | | | |
| Post-treatment | | | | | |
| Responders W30 | | | | | |
| W36 | | | | | |
| W42 | | | | | |
| W48 | | | | | |
| W60 | | | | | |
| W72 | | | | | |

**We need all demographic data (age, sex and race) and viral load.**

**Samples are restricted to HCV genotype 1a.**

**This is for the Specific Aim 1.**

**Protocol Part III: Sample Requirements. 20 patients with SVR (genotype 2a)**

| Visit | Liver# patients, mm* | Blood# patients, ml | DNA# patients, ug | Liver Biopsy Slides# patients, slides/patient | Other (describe)# pts, amount |
|---|---|---|---|---|---|
| Screen 1 | | | | | |
| Screen 2 | | | | | |
| Baseline | | 0.5 ml x 20 pts | | | |
| Lead in Week 4 | | | | | |
| Week 8 | | | | | |
| Week 12 | | | | | |
| W16 | | | | | |
| Week 20 | | | | | |
| Week 24 | | | | | |
| Randomized Month 9 | | | | | |
| Month 12 | | | | | |
| Month 15 | | | | | |
| Month 18 | | | | | |
| Month 21 | | | | | |
| Month 24 | | | | | |
| Month 27 | | | | | |
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| Month 33 | | | | | |
| Month 36 | | | | | |
| Month 39 | | | | | |
| Month 42 | | | | | |
| Month 45 | | | | | |
| Month 48 | | | | | |
| Post-treatment | | | | | |
| Responders W30 | | | | | |
| W36 | | | | | |
| W42 | | | | | |
| W48 | | | | | |
| W60 | | | | | |
| W72 | | | | | |

**We need all demographic data (age, sex and race), viral load and genotypes.**

**Samples are restricted to HCV genotype 2a.**

**This is for the Specific Aim 2.**

**Protocol Part III: Sample Requirements. 20 patients with relapse response (genotype 1a)**

| Visit | Liver# patients, mm* | Blood# patients, ml | DNA# patients, ug | Liver Biopsy Slides# patients, slides/patient | Other (describe)# pts, amount |
|---|---|---|---|---|---|
| Screen 1 | | | | | |
| Screen 2 | | | | | |
| Baseline | | 0.5 ml x 20 pts | | | |
| Lead in Week 4 | | | | | |
| Week 8 | | | | | |
| Week 12 | | | | | |
| W16 | | | | | |
| Week 20 | | | | | |
| Week 24 | | | | | |
| Randomized Month 9 | | | | | |
| Month 12 | | | | | |
| Month 15 | | | | | |
| Month 18 | | | | | |
| Month 21 | | | | | |
| Month 24 | | | | | |
| Month 27 | | | | | |
| Month 30 | | | | | |
| Month 33 | | | | | |
| Month 36 | | | | | |
| Month 39 | | | | | |
| Month 42 | | | | | |
| Month 45 | | | | | |
| Month 48 | | | | | |
| Post-treatment | | 0.5 ml x 20 pts at the time of first appearance of the relapse | | | |
| Responders W30 | | | | | |
| W36 | | | | | |
| W42 | | | | | |
| W48 | | | | | |
| W60 | | | | | |
| W72 | | | | | |

**We need all demographic data (age, sex and race) and viral load.**

**Samples are restricted to genotype 1a**

**This is for the Specific Aim 3.**