Pre-study Serial Histology Snap Shot

Sites Participating: All sites except NIDDK (Site #20); VCU will be acting as the Coordinating Center

Principal Investigator: Mitchell Shiffman, MD (Virginia Commonwealth University)

Separate Consent Form: Yes

Withdrawal Form: No

Eligible Patients: All HALT-C Participants

Forms to be filled out:

By patient and site Study Coordinator at Screening: Informed consent, release of medical information statement, and Form #160 Documentation of Prior Liver Biopsy

By VCU staff and Pathology Center: Shipping logs

By Pathology Center: Form #161 Serial Histology Biopsy Review

Flow of Operations:

- Patient signs informed consent for PSSH
- Patient will provide name and location of hospital/facility where previous liver biopsies were performed
- Patient will sign a “release of medical information statement” for each biopsy performed prior to their entrance into HALT-C
- Signature page of informed consent, list of previous liver biopsies and locations, and signed “release of medical information statement should be faxed and then mailed to VCU
- VCU will contact hospital(s) and obtain previous liver biopsy slides for patient
- Biopsy slides will be blinded at VCU
- Biopsy slides will be sent to the Pathology Center for review
- Biopsy slides will be sent back to VCU from the Pathology Center
- VCU will return slides to originating hospital

NOTE: Massachusetts General Hospital and St. Louis University will be retrieving and blinding their patients’ pre-study biopsy slides per IRB instructions. The slides will be reviewed by the Pathology Center and then returned to either MGH or SLU who will then return them to the originating hospital.

Pre-study Serial Histology as a Predictor of Response to Continuous Therapy during the HALT-C Trial

Principal Investigator: Mitchell Shiffman, M.D.

I. PURPOSE OF THE STUDY/DESCRIPTION

In virologic non-responders to prior therapy:

1. Patients will be divided into cohort A (minimal decrease in inflammatory activity) and cohort B (decreased inflammatory activity) based on prior treatment responses as assessed by: 1) serum AST/ALT (SGOT/SGPT) during treatment and 2) liver biopsy within 6 months of stopping treatment.
2. Determine the individual rate of histologic progression from serial biopsies in the above two cohorts.
3. Correlate inflammatory treatment response with the rate of fibrosis from serial pre-study liver biopsies.
4. Identify a group of patients with rapid progression in histologic fibrosis despite therapy.
5. Determine whether these cohorts defined on pre-study response to interferon predicts the rate of progression in fibrosis, inflammatory improvement and outcome during continuous therapy.

Estimated number of patients from all NIH HALT-C participating sites: 400. Thus, a pre-study response in 200 patients under observation and 200 on continuous therapy can be evaluated.

II. INCLUSION CRITERIA:

1. Eligible for the main HALT-C protocol
2. Willing to participate in this study by signing an informed consent and a medical release form to obtain prior biopsy liver tissue.
3. Three (3) or more data points defined below:
   a. 3 prior biopsies performed, any 2 being 5 years apart and this may include a biopsy performed at the screening visit **or**
   b. 2 biopsies and a well established initial exposure (e.g. blood transfusion, followed by a NANB acute hepatitis)
4. At least 2 adequate biopsies as defined below:
   a. >2 cm in length or
   b. ≥1 cm in length with ≥3 portal areas in a clear cut stage

(An inadequate biopsy is one that is <1 cm in size or any size biopsy that is deemed inadequate for staging. A biopsy inadequate for staging may be helpful in grading inflammation.)

III. SCHEDULE OF VISITS AND SPECIMEN COLLECTION:

At the screening visit the patient will be consented and sign a pathology material release authorization form. If participating centers do not have a standard medical release form, one is included on page 9 of this section. Informed consent will be included in the main Halt –C Trial consent form with a statement similar to that below:
"We give permission to obtain and evaluate all prior biopsy material. My confidentiality will be preserved similar to all other information in this study." Those patients who are not eligible for the main HALT-C protocol will not be eligible for this ancillary study.

During Screening (S00), by medical chart review, patient interview, and calling the original site where the prior biopsy is, the Study Coordinator:

1. Completes the Documentation of Prior Liver Biopsy, Form # 160, detailing the information listed below for each prior biopsy: For each previous biopsy , not including the biopsy used for screening for entry into HALT-C record the following information:

a. The Coordinator records the dates of prior liver biopsy, starting with the most recent, in the month/year (MM/YYYY) format.
b. Records the sponsor and title and number of the protocol if the prior biopsy is part of a previously sponsored protocol (who is the sponsor: Schering, Roche, Amgen, other etc. and if possible the title and number of the protocol). Or record the name and address of the hospital the prior biopsy is located. Or record "Onsite" if the prior biopsy is located at the clinical center's institution.
c. Records the physician's name and phone number and /or beeper number. This will be instrumental when the Central Biopsy Coordinator at Virginia Commonwealth Health System attempts to retrieve the prior biopsy.
d. Record 1 for Pre-treatment prior biopsy and record 2 for Post-treatment prior biopsy.
e. Record the date prior interferon treatment began in the month/year (MM/YYYY) format.
f. Record the date prior interferon treatment ended in the month/year (MM/YYYY) format.
g. Record the Baseline Serum ALT or AST in U/L. Or record Not Available.
h. Circle if this result was for ALT or AST determination.
i. Record the date this assay was performed.
j. Record the End of Treatment Serum ALT or AST in U/L. Or record Not Available.
k. Record if this result was for ALT or AST determination.
l. Record the date this assay was performed.
m. Record the end of Follow-up Serum ALT or AST in U/L. Or record Not Available.
n. Record if this result was for AST or ALT determination.
o. Record the date this assay was performed.
p. Record 1 if there is a request to return /ship the prior biopsy back to its original location. Record 2 if there is not a request to return the prior biopsy.
q. Record 1 if the patient signed a Medical Release Form. Record 2 if the patient did not sign a medical release form.
r. Record 1 if you are able to retrieve the prior biopsy. Record 2 if you are not able to retrieve the prior biopsy.
s. If not able to retrieve the prior biopsy record the reason why using the code box on the last page of the form.
t. Record the number of slides available for shipment to UC Irvine.
u. Record the number of blocks available for shipment to UC Irvine.

2. Study coordinators should attempt to obtain biopsy slides **and** paraffin blocks for all previous biopsies during the six week screening period. These materials should be re-labeled with HALT-C Trial ID label and set aside for shipment to Virginia Commonwealth University Health System.

3. Data enter completed Form #160 Documentation of Prior Liver Biopsy into the Halt-C Trial Data Management System.

4. Fax this completed form to the Central Biopsy Coordinator at UCIMC. (See Contact Information, section VI). Prior to Faxing this form, please write the names of patients and any other required identifying information for the biopsies that you were not able to obtain. **Please note:** This identifying information should not be sent to the DCC or be included in the patient's HALT-C Trial study notebook.

5. Those biopsy slides that could not be retrieved by the study coordinator at the site will be attempted to be retrieved by the Central Biopsy Coordinator at VCUHS. All pertinent information should be provided to the VCUHS Biopsy Coordinator in order to increase the retrieval rate. CALL the VCUHS Biopsy Coordinator with the patient's name, Social Security number, date of birth, and the Trial ID number.

6. As part of the main Trial, a complete medical history with presumable date of HCV acquisition will be obtained. Form # 6 Baseline History.

IV. SPECIMEN COLLECTION:

There is no additional specimen collection, except for the previous biopsy material retrieval (H&E and Trichrome-stained slides and paraffin blocks for further processing).
All study participants should have adequate biopsy specimens as defined below:

- >2 cm in length or
- ≥1 cm in length with ≥3 portal areas in a clear cut stage

V. SHIPPING AND HANDLING:

Study Coordinators should complete the Pre-study Serial Histology Shipping Log, recording information for each biopsy obtained. This shipping log is located on page 10 of this document. **This shipping log should not be data entered**. A copy should be included in the shipping container used to ship slides and blocks to VCUHS. It should also be Faxed to VCUHS prior to shipping.

Using the provided mailing labels, each site will ship the slides/paraffin blocks with all the accompanying identifying information and the shipping log **once a month** via FedEx service (FEDEX account number = 2504-9389-4) to:

Carolyn Frey
VCUHS
1200 East Broad St.
14th Floor East Wing, Rm 16
Richmond, VA 23219
Telephone # (804) 828-9153
Fax # (804) 828-4945
E-Mail: cafrey@mail2.vcu.edu

Shipping should be done in slide containers placed in bubble bag packages. Slide boxes will be supplied by UC Irvine.

Once received, stained slides will be logged in and stored in special slide libraries and paraffin blocks will be processed by Pathology to obtain H&E and trichrome-stained slides at UCIMC. Note: The original slides (H&E and Trichrome are the original resource, blocks will be processed to obtain slides only if these slides are unavailable or the material is inadequate. UCIMC will keep the appropriate biopsy material log with all the information, including the shipping status. All biopsy slides will then be forwarded to the Data Coordinating Center (NERI) to be blinded and then forwarded to AFIP (Dr. Z. Goodman) for final reading by the Central Pathology Committee.

VI. Contact Information:

Carolyn Frey
VCUHS
1200 East Broad St.,
14th Floor East Wing, Rm 16
Richmond, VA 23219
Telephone # (804) 828-9153
Fax # (804) 828-4945
E-Mail: cafrey@mail2.vcu.edu

Staining and Processing procedures to be followed at VCUHS:

VII. MASSONIS TRICHROME FOR CONNECTIVE TISSUE

Arrange the tissue in a serpentine-like manner on a filter paper in a special cassette and drop the cassette in B5 fixative for 5 minutes before it is delivered to Pathology for further processing.

1. PRINCIPLE

These techniques are sequence procedures employing plasma stain, followed by a phosphotungstic or phosphomolybdic acid mixture, followed by a collagen-fiber stain. Staining is done at an acid pH to increase selectivity for the collagen fiber.

Acid dyes are used to stain the acidophilic cytoplasm and muscle fibers; Biebrich scarlet is a common example. Collagen fibers are also acidophilic but, on the final result, color differently from the muscle and cell cytoplasm because of the action of the phosphomolybdic (or phosphotungstic) acid. One theory of why this is so suggests that the acid is taken up by the connective tissue and then replaced by aniline blue or similar dyes. Aniline blue is more of a group of related dyes rather than a single dye, but the group is considered to act as an acid dye. The mechanism would act as a substitution reaction of one acid for another.

Studies have shown that the polymeric and pentavalent phosphomolybdic and phosphotungstic acids actually become located in the fibers that are stained selectively by the trichrome methods. These structures are rich in basic groups. The phosphomolybdic and phosphotungstic acids have more acidic groups than the substrate tissue has basic groups. As a result numerous acidic groups are left free to bind dyes that are rich in basic groups. The phosphomolybdic or phosphotungstic acid thus acts as a link connecting basic groups of the connective tissue fiber to the basic groups of the dye. The phosphomolybdic or phosphotungstic acid treatment has the ultimate effect of making an amphoteric dye that would ordinarily act a an acid dye and act as a basic dye.

Collagen is readily entered by almost any dye, as it is of comparatively loose texture. Cytoplasm has lesser permeability and is more selective to a dye. In Masson's trichrome, the acid plasma stain (Biebrichscarlet-acid fuchsin solution) enters all the acidophilic tissue constituents including the collagen. Because of the lesser permeability of the cytoplasm, it will remain there even when the sections are exposed to the action of the phosphomolybdic and phosphotungstic acid. Acid causes the Biebrich scarlet to diffuse cut of the collagen, and one purely physical reason may be that the loose texture of the collagen aids in dye diffusion, pH factors may also pay a role, as the pH may not be sufficiently acidic to give selective collagen staining with the first treatment of Biebrich scarlet. There is no chemical combination with the Biebrich scarlet, the dye will easily diffuse out on subsequent treatments, enabling it to be differentially stained with the aniline blue on the chemical basis method.

2. SPECIMEN

Bouin's fixed tissue is preferable; 10% Neutral buffered formalin may be used. Tissues are then processed and sectioned at 2-3 microns (renal), 5-6 microns for heart biopsies and etc.

3. REAGENTS:

a. **BOUIN' S SOLUTION**

Picric Acid, Saturated aqueous solution	75.0 (cc) ml
Formaldehyde 37-40%	25.0 (cc) ml
Glacial acetic acid	5.0 (cc) ml

b. **WEIGERT'S IRON HEMATOXYLIN SOLUTION**

SOLUTION A

Hematoxylin	1.0 gm
95% alcohol	100.0 (cc) ml

SOLUTION B

Ferric Chloride, 29%	4.0 (cc) ml
Aqueous Distilled Water	95.0 (cc) ml
Hydrochloric acid	1.0 (cc) ml

NOTE: Always add the acid to the solution will splash or react if not done properly

WORKING SOLUTION

Add equal parts of Solution A (20 ml) and Solution B (20 ml) in a coplin jar.

c. **BIEBRICH SCARLET-ACID FUCHSIN SOLUTION**

Biebrich scarlet (1 gm in 100 ml of distilled water) 1%	90.0 (cc) ml
Acid Fuchsin (1 gm in 100 ml of distilled water) 1%	10.0 (cc) ml
G1acia1 acetic acid	1.0 (cc) ml

d. **PHOSPHOMOLYBDIC-PHOSPHOTUNGSTIC ACID SOLUTION**

Phosphomolybdic acid	5.0 gm
Phosphotungstic acid	5.0 gm
Distilled water	200.0 (cc) ml.

e. **ANILINE BLUE SOLUTION** (in the heart biopsies the Sigma brand is used)

Aniline blue	2.5 gm
Glacial acetic acid	2.0 (cc) ml
Distilled water	100.0 (cc) ml.

f. **2% LIGHT GREEN SOLUTION**

Light green	5.0 gm
Distilled water	250.0 (cc) ml
Glacial acetic acid	2.0 (cc) ml

Heat water and dissolve light green, cool filter and add acid·

g. **1% ACETIC ACID WATER SOLUTION**

Glacial acetic acid	1.0 (cc) ml
Distilled water	100.0 (cc) ml

h. Histoclear

i. Cytoseal

4. QUALITY CONTROL

Positive controls are processed and stained with specimen and evaluated by the histology staff before given to pathologist for interpretation. If staining is unsatisfactory it must be repeated. Any such corrective action is recorded on stain maintenance and quality control record in histology.

5. STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's Solution for 1 hour at room temperature, if formalin fixed.
3. Wash in running water until yellow color appears.

4. Rinse in distilled water.
5. Place slides into Weigert's Hematoxylin for 10 minutes.
6. Wash in Running water for 10 minutes.
7. Rinse in distilled water.
8. Place slides into the Biebrich scarlet-acid fuchsin solution for 2 minutes.
9. Rinse in distilled water.
10. Place slides into phosphomolybdic-phosphotungstic solution for 10-12 minutes.
11. Place slides into the aniline blue solution for 4-5 minutes.
12. Rinse in distilled water.
13. Place slides in 1% glacial acetic acid for 3 minutes.
14. Dehydrate and clear in histoclear.
15. Mount with Cytoseal.

6. RESULTS:
   1. Nuclei - black
   2. Cytoplasm - keratin, muscle fibers and intercellular fibers - red
   3. Collagen, mucin - blue

7. REFERENCE

Masson, P.J., J. Tech. Methods, Volume 12:75-90, AFIP Modification, 1929
Sheehan, D.C., and Hrapchak, B.B., Theory and Practice of Histotechnology, 2nd ed., St. Louis, C.V. Mosby Co., 1980, pp 189191

VIII. HEMATOXYLIN AND EOSIN (HARRIS)

Arrange the tissue in a serpentine-like manner on a filter paper in a special cassette and drop the cassette in B5 fixative for 5 minutes before it is delivered to Pathology for further processing.

1. PRINCIPLE**:**

Hematoxylin, a natural dye which was first used about 1863, is the most valuable stain used in histology. In combination with aluminum, iron, chromium, copper or tungsten salts it is a powerful nuclear stain. The active coloring agent, hematein, is formed by the oxidation of hematoxylin. The most common formulas for staining with hematoxylin are the combinations with aluminum in the form of alum. Those in general use were formulated by Harris, Mayer, Lillie, etc. Sections stained with hematoxylins may be counterstained with Eosin, Safran, Phloxine or other contrasting stains.

Anyone who has worked with the H&E stain knows that many factors constitute to cause some variation in this technique.
Examples of this are the fixative used, fixation length, age of staining solution, etc.

There are 2 methods of staining when hematoxylin is used: Progressive and Regressive.

Progressive staining is accomplished by employing a hematoxylin solution which contains an excess of aluminum salts or acid, increasing the selectivity for nuclei. After staining with hematoxylin, the slides are washed well in water and the secondary stain applied.

In regressive staining, the sections are overstained in a neutral solution of hematoxylin, then the sections are neutralized with an alkaline solution such as weak ammonia or lithium carbonate water. In a well differentiated section the cytoplasm should be colorless and nuclear substances should be clearly visible.

Counterstains for hematoxylin are a matter of personal preference. They should be considered a secondary stain in the case of H&E staining.

2. SPECIMEN

Tissue fixation may be used with any fixation.

Paraffin or frozen technique can be used. Cut paraffin sections are 5 microns.

3. REAGENTS:

HARRIS HEMATOXYLIN

Hematoxylin crystals	5.0 gms
Alcohol, 100%	50.0 mls
Ammonium or potassium alum	100.0 gms
Distilled water	1000.0 mls
Mercuric oxide (red)	2.5 gms

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible. (limit this heat to less than 1 minute and stir often). Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat. The stain is ready for use as soon as it cools. Addition of 2-4 m of glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. Filter before use. Label with expiration date.

Where to obtain materials:

- Hematoxylin Crystals - Aldnior 86, 118-9 CI# 75290 Aluminum Ammonium Sulfate 12 Hydrate - Baker 1-0484
- Mercuric Oxide (red) Baker 2620
- Eosin Y - SIGMA E4009

4. STOCK EOSIN SOLUTION

Eosin Y, water soluble	2.0 gms
Distilled water	160.0 mls
Alcohol, 95%	640.0 mls

Dissolve Eosin Y in the distilled water, then add the 95% alcohol. If a deeper shade is desired, add a drop of acetic acid to each 100 cc of solution. Label with expiration date.

5. WORKING EOSIN PHLOXINE SOLUTION

Stock Eosin Solution	280.0 ml
Isopropyl Alcohol, absolute	840.0 ml
1.5% aqueous Phloxine	30.0 ml

6. 1.5% AQUEOUS PHLOXINE SOLUTION

Phloxine	1.5 gms
Distilled water	100.0 cc

7. ACID ALCOHOL

70% Isopropyl Alcohol	1000.0 cc
Hydrochloric Acid, concentrated	10.0 cc

8. 1% SODIUM BORATE SOLUTION

Sodium Borate	1.0 gms
Distilled Water	100.0 ccs

9. QUALITY CONTROL

Pathologist evaluates the stain for quality and incorporates this information in the pathology report.

10. PROCEDURE:

1. Hemo De, absolute alcohol, water
2. If sections are Zenker fixed, treat with either Lugol's solution or 1% alcoholic iodine solution, followed by 5% Sodium Thiosulfate to remove mercuric crystals.
3. Hematoxylin 4 minutes
4. Wash in Distilled water
5. Differentiated in 1% acid alcoholic until red-brown in color approximately 5 dips
6. Wash in water
7. Blue in Sodium Borate for 2 minutes
8. Wash well in water
9. Working Alcoholic Eosin-Phloxine stain for 10 dips.
10. Rinse in 85% Isopropyl Alcohol
11. Dehydrate and Clear in Hemo De
12. Mount in cytoseal

11. RESULTS

Nuclei stain blue
Cytoplasm stain pink

12. REFERENCES:

ARMED FORCES INSTITUTE OF PATHOLOGY MANUAL, Page 38.

MEDICAL INFORMATION RELEASE AUTHORIZATION

PATIENTS **PLEASE FILL OUT THIS FORM AND SEND TO YOUR DOCTOR AND/OR GASTROENTEROLOGIST**

I Hereby Request From:

_____

_____

_____

To Furnish To: **Mitchell Shiffman, MD**
from the Virginia Commonwealth University Health System the following information:

Specific Information Requested
MEDICAL HISTORY/ LABORATORY REPORTS/ PATHOLOGY MATERIAL AND REPORTS

_____

_____

Patient's Name:
_____

DOB:
_____

Patient HALT-C Trial ID#:
_____

_____
Patient's Signature and Today's Date

DOCTORS PLEASE FORWARD MEDICAL INFORMATION FOR THE ABOVE PATIENT TO :

Carolyn Frey
VCUHS
1200 East Broad St.,
14th Floor East Wing, Rm 16
Richmond, VA 23219
or
PLEASE FAX TO (804)-828-4945

THANK YOU FOR YOUR COOPERATION.

HALT-C Trial

Pre-study Serial Histology AS Shipping Log #1

DO NOT DATA ENTER THIS LOG **Version: 6/15/00**

Section A: General Information

A1. Site Name: _____

A1. Shipping Date : MM / DD / YYYY __ __ / __ __ / __ __ __ __

A2. Initials of Person Completing Form: __ __ __

A3. Number of Boxes in Shipment: _____

A4. FedEx Tracking # _____

	To be completed at the Clinical Site Prior to shipping to UC Irvine					
	Patient ID	Patient Initials	Biopsy Date *(MM/YYYY)*	# Slides	# Blocks	Slide Box #
	a.	b.	c.	e.	f.	g.
1	__ __ - __ __ __ - __	__ __ __	__ __/__ __ __ __	__ __	__ __	___
2	__ __ - __ __ __ - __	__ __ __	__ __/__ __ __ __	__ __	__ __	___
3	__ __ - __ __ __ - __	__ __ __	__ __/__ __ __ __	__ __	__ __	___
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HALT-C Trial

Pre-study Serial Histology AS Shipping Log #2

DO NOT DATA ENTER THIS LOG **Version: 6/15/00**

Section A: General Information

A1. Shipping Date : MM / DD / YYYY __ __ / __ __ /__ __ __ __

A2. Initials of Person Completing Form: __ __ __

A3. Number of Boxes in Shipment: _____

A4. FedEx Tracking # _____

	To be completed at the UC Irvine Prior to shipping to NERI					
	Patient ID	Patient Initials	Biopsy Date *(MM/YYYY)*	# Slides H & E	# Slides Trichrome	Slide Box #
	a.	b.	c.	e.	f.	g.
1	__ __ - __ __ __ - __	__ __ __	__ __/__ __ __ __	__ __	__ __	___
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