Serum Fibrosis Markers Snap Shot

Sites Participating: University of Massachusetts (Site #11 UMASS) / University of Connecticut (Site #11UCONN), Massachusetts General Hospital (Site #13 MGH) University of Michigan (Site #18 UMICH), Virginia Commonwealth University (Site #19 VCU)

Principal Investigator: Robert Fontana, MD (University of Michigan)

Co-Investigators: Anna Lok, MD; Zachary Goodman, MD; Mitchell Shiffman, MD; Jules Dienstag, MD; Herb Bonkovsky, MD; and Savant Mehta, MD

Eligible Patients: Lead-in, W20 Responders, Randomized, Breakthrough/Relapser, Express[1]

Study Name: SERUM FIBROSIS MARKERS IN CHRONIC HEPATITIS C ANCILLARY STUDY

Separate Consent Form: No

Withdrawal Form: No

Visit Schedule (additional data/specimens and forms for AS)

Note: "**X**" means all participating sites take part.

Lead-In Patients

Visit Number ➔	Form #	S00	W00	W24
Serum TGF-β1[1]	100		18	18
Hepatic TGF-β1 mRNA[2]	101	18		
Serum Fibrosis Aliquot Form[3]	102		11,13,19	11,13,19
Serum YKL-40[4]	103		**X**	**X**
Serum PIIINP[5]	104		**X**	**X**
Serum TIMP-1[5]	105		**X**	**X**
Specimen Collection Log[5]	503		18	18
Liver biopsy	14	18		

[1] 100 Serum TGF-b1 mRNA only Site 18 UMICH
[2] 101 Hepatic TGF-b1 mRNA only Site 18 UMICH
[3] 102 Serum Fibrosis Aliquot Form only Sites #11 UMASS/UCONN, #13 MGH, #19 VCU
[4] YKL-40, PIIINP, TIMP-1 in all study patients
[5] 503 Shipping Log only Site 18 UMICH

[1] Specimens for Express patients are collected and tested at Month 12, Month 24, Month 36 and Month 48

Week 20 Responders

Visit Number ➔	Form #	W48	W72
Serum TGF-β1[1]	100	18	18
Hepatic TGF-β1 mRNA[2]	101		
Serum Fibrosis Aliquot Form[3]	102	11, 13,19	11, 13, 19
Serum YKL-40[4]	103	**X**	**X**
Serum PIIINP[5]	104	**X**	**X**
Serum TIMP-1[5]	105	**X**	**X**
Specimen Collection Log[5]	503	18	18
Liver biopsy	14		

Randomized Phase

Visit Number ➔	Form #	R00 BT/R	M12	M24	M36	M48
Serum TGF-β1[1]	100	18	18	18	18	18
Hepatic TGF-β1 mRNA[2]	101			18		18
Serum Fibrosis Aliquot Form[3]	102	11, 13,19	11, 13,19	11, 13,19	11, 13,19	11, 13,19
Serum YKL-40[4]	103	**X**	**X**	**X**	**X**	**X**
Serum PIIINP[5]	104	**X**	**X**	**X**	**X**	**X**
Serum TIMP-1[5]	105	**X**	**X**	**X**	**X**	**X**
Specimen Collection Log[5]	503	18	18	18	18	18
Liver biopsy	14			18		18

[1] 100 Serum TGF-b1 mRNA only Site 18 UMICH
[2] 101 Hepatic TGF-b1 mRNA only Site 18 UMICH
[3] 102 Serum Fibrosis Aliquot Form only Sites #11 UMASS/UCONN, #13 MGH, #19 VCU
[4] YKL-40, PIIINP, TIMP-1 in all study patients
[5] 503 Shipping Log only Site 18 UMICH

Hyaluronic Acid Visit Schedule

Note: "**X**" means all participating sites take part.

Lead-In Phase or Responder Phase

Visit Number ➔	S00	W00	W02	W04	W08	W12	W16	W20	W24	W48	W72
Stored sample (#111 or #123)		**X**							**X**	**X**	**X**

Randomized Phase

Visit Number ➔	R00	M09	M12	M15	M18	M21	M24	M27	M30	M33	M36	M39	M42	M45	M48	M54
Stored sample (#111 or #123)	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	

Note: No additional specimens are collected for this study. Tests are performed on specimens stored for the main trial (seq.#111 or seq.#123). Results are maintained in a separate data file and are not included in the main HALT-C database.

Visit Schedule For Additional Tests Performed at WAKO

Additional tests included in the Serum Fibrosis Protocol are:

1. Direct Bilirubin
2. Total bile acids
3. GGTP
4. C-reactive protein
5. Total Cholesterol
6. HDL cholesterol
7. Rheumatoid factor
8. Transferrin
9. Unsaturated iron binding capacity (UIBC)

Lead-In Phase or Responder Phase

Visit Number →	S00	W00	W02	W04	W08	W12	W16	W20	W24	W48	W72
Stored sample (#111 or #123)		X							X	X	X

Randomized Phase

Visit Number →	R00	M09	M12	M15	M18	M21	M24	M27	M30	M33	M36	M39	M42	M45	M48	M54
Stored sample (#111 or #123)	X		X		X		X		X		X		X		X	

Note: No additional specimens are collected for this study. Tests are performed on specimens stored for the main trial (seq.#111 or seq.#123). Results are maintained in a separate data file and are not included in the main HALT-C database.

Serum Fibrosis Markers in Chronic Hepatitis C Ancillary study

Principal Investigator: Robert Fontana, MD

Co-Investigators: Anna Lok, MD; Zachary Goodman, MD; Mitchell Shiffman, MD; Jules Dienstag, MD; Herb Bonkovsky, MD; and Savant Mehta, MD

I. Introduction

The objective of the NIH HALT-C trial is to determine if long-term interferon (IFN) therapy can reduce hepatic fibrosis and progression to cirrhosis. Hepatic fibrosis will be assessed by semi-quantitative scoring (Ishak) and computerized morphometry of serial liver biopsies (years 0,2, and 4). However, liver biopsy is expensive and carries a small but definite risk of complications. In addition, sampling errors may occur. The aim of this ancillary study is to evaluate the reliability of a panel of serum markers: N-terminal propeptide collagen III (PIIINP), hyaluronic acid (HA), YKL-40 (refer to Amendment #1), TIMP-1 (refer to Amendment # 2), and TGF-β1 in predicting hepatic fibrosis. In addition, we will determine the effects of long-term IFN therapy on these makers and determine if changes in these serum markers correlate with changes in hepatic fibrosis. Blood will be collected from patients in 4 participating centers at baseline (W00), week 24 (W24), week 48 (W48), week 72 (W72), month 12 (M12), month 24 (M24), month 36 (M36), and month 48 (M48) to test for serum PIIINP, HA, and TGF-β1 (serum and hepatic TGF-β1) will be performed only at UMICH) and other serum markers of hepatic fibrosis as additional assays become available during the course of this study (YKL-40, TIMP-1). All liver biopsies from one site (University of Michigan) will also be tested for TGF-β1 mRNA expression. This study will consist of a cross-sectional and longitudinal component. In the cross-sectional study, correlation between hepatic fibrosis in Screening (S00) liver biopsies and serum PIIINP, HA, and TGF-β1 will be determined. In the longitudinal study, correlations between changes in each serum marker and changes in hepatic fibrosis will be determined and compared in the IFN treated and untreated control groups. The large number of patients, the long duration of treatment, and the serial liver biopsy samples in this study that will be scored using sensitive quantitative measurements of hepatic fibrosis will enable us to determine if any of these serum makers reliably correlate with hepatic fibrosis in patients with chronic hepatitis C and to determine if they can be used to monitor response to IFN therapy.

II. Background

The natural history and long term prognosis in chronic hepatitis C is closely linked to the development of hepatic fibrosis (1,2). Recent studies suggest that IFN therapy may have anti-fibrotic effects in addition to anti-viral and immunomodulatory effects. However, conclusive data are lacking due to the small size of the studies, the short duration of treatment and follow-up, and the difficulties in quantifying hepatic fibrosis. Most studies have used semi-quantitative scoring systems with a narrow range for fibrosis: 3 scales for Knodell and 4 for Metavir. These systems are insensitive to small changes in hepatic fibrosis. Using more sensitive morphometric methods to measure quantitative hepatic collagen (QHC) content, Manabe et al found that QHC content significantly decreased in 59 patients after 6 months of IFN even though the Knodell fibrosis scores remained unchanged (4). In addition, QHC content increased in untreated controls. Thus, more sensitive methods of determining hepatic fibrosis with a broader range of values are needed to assess the anti-fibrotic effect of IFN in hepatitis C. Previous studies demonstrate a strong correlation between morphometric measurements of QHC content and liver hydroxyproline content and a high degree of reproducibility suggesting that computerized morphometry may be a more appropriate method of assessing the anti-fibrotic effects of IFN (5).

Although liver biopsy is the best established means of assessing disease activity and severity in chronic hepatitis C, liver biopsy is expensive and associated with a small but definite risk of complications. In addition, sampling errors may occur making reliable assessment of liver

damage difficult, particularly regarding the extent of hepatic fibrosis (6). As a result, sensitive and reliable serological assays for the non-invasive detection and monitoring of hepatic fibrosis are needed. Serum PIIINP, HA, YKL-40, TIMP-1 (refer to Amendments 1 and # 2), and TGF-β1 levels are the most extensively studied markers of hepatic fibrosis in chronic hepatitis C (7).

III. Hypotheses/ Aims

The aim of this ancillary study is to evaluate the reliability of a panel of serum markers: N-terminal propetide collagen III (PIIINP), hyaluronic acid (HA), YKL-40, TIMP-1 (refer to Amendment # 1 and # 2), and TGF-β1 in predicting hepatic fibrosis in chronic hepatitis C. In addition, we will determine the effects of long-term IFN therapy on these markers and if changes in these serum markers correlate with changes in hepatic fibrosis.

The hypotheses to be tested in this study are:

1. Serum levels of PIIINP, HA, and TGF-β1, singly or in combination, correlate well with hepatic fibrosis in patients with chronic hepatitis C.
2. Changes in serum levels of PIIINP, HA, and TGF-β1 during the course of the HALT-C trial correlate with changes in hepatic fibrosis in patients with chronic hepatitis C.
3. Long-term IFN therapy induces significant decrease in hepatic fibrosis as well as serum levels of PIIINP, HA, and TGF-β1 in patients with chronic hepatitis C even in the absence of a virologic response.

IV. Methods

Patients will be enrolled at University of Michigan (UMICH), University of Massachusetts (UMASS)/University of Connecticut (UCONN), Virginia Commonwealth University (VCU), and Massachusetts General Hospital (MGH). An overview of the proposed study is listed below.

Study Visit	S00	W00	W24	W48**	W72**	M12	M24	M36	M48
Serum fibrosis markers *		X	X	X	X	X	X	X	X
Liver biopsy ***	X						X		X

* PIIINP, HA, TIMP in 360 patients and TGF-β1 in 90 University of Michigan (site 18)patients only.
** in Week 20 responders
*** QHC and Ishak scoring in 360 patients (site 11, 13, 18 and 19) and hepatic TGF-β1 mRNA in 90 University of Michigan (site 18) patients only.

A. Hepatic fibrosis

All liver biopsies obtained at Screening (S00), month 24 (M24), and month (M48) will be reviewed and scored for inflammation (0-6) and fibrosis (0-6) by the HALT-C Pathology Committee using the Ishak scoring system (8). Quantitative hepatic collagen (QHC) content will be determined using sirius red staining with computerized morphometry with an anticipated range of 5-40% corresponding to Ishak fibrosis scores of 3 to 6 (5). Changes in semi-quantitative Ishak fibrosis (IF) and inflammation (IN) scores and QHC between Screening (S00) to month 24 (M24), month 24 (M24) to month 48 (M48), and Screening (S00) to month 48 (M48) will be calculated for each patient with adequate liver samples at all three time points.

B. Serum fibrosis markers

- **PIIINP**- PIIINP will be tested using a commercially available rapid equilibrium RIA that detects intact PIIINP antigens using polyclonal antibodies manufactured by Orion Diagnostics (9,10).

- **Hyaluronic acid-** HA levels will be tested using a commercially available RIA kit (Pharmacia AB Upsula, Sweden) (11,12).
- **YKL-40,** YKL-40 will be tested using a commercially available YKL-40 ELISA format test kits designed to measure YKL-40 concentrations in serum provided by the manufacturer (Quidel Corporation, San Diego CA)(refer amendement#1)
- **TIMP-1** -TIMP-1 will be tested using a commercially available TIMP-1 immunoassay ELISA kit designed to measure total TIMP-1 concentrations in serum and plasma

  (Quantikine - R & D Systems, Minneapolis, MN).(refer amendment#2)
- **TGF-β1-** TGF-β1 levels will be tested in University of Michigan (site18) patients only using a standard sandwich ELISA assay with antibodies and standards obtained from Pharmingen, San Diego, CA (13).

The markers that will be tested and the assays that will be performed are selected based upon current knowledge and assay availability. Additional or alternative markers may be tested as new assays become available.

PIIINP, Hyaluronic Acid, YKL-40, TIMP-1 (refer to Amendment #1 and 2)

1. Inclusion/ exclusion

   Blood samples from all **540** participants enrolled at 4 centers (University of Massachusetts / University of Connecticut, Massachusetts General Hospital, University of Michigan, Virginia Commonwealth University) will be collected.

   135 patients / center
   45 week 20 (W20) responders followed through week 72 (W72)
   90 randomized non-responders

2. Visit schedule

   90 randomized non-responders (n=360 total)
   Baseline (W00), week 24 (W24), month 12 (M12), month 24 (M24), month 36 (M36), and month 48 (M48)

   45 Week 20 (W20) responders (n= 180 total)
   Baseline (W00), week 24 (W24), week 48 (W48), week 72 (W72)

3. Specimen Collection + Processing

   a. Serum:
      1. 20 ml of whole blood should be collected in a single Red topped tube at W00, W24,M12, M24, M36, M48 and at W48 + W72 for Week 20 responders.
      2. Tubes should be centrifuged (3000 rpm X 15 min) to separate serum.
      3. MGH, UMASS and VCU only:
         The serum must be aliquotted into 10 tubes with 0.5ml and 5 tubes with 1.0ml. The aliquot tubes supplied by the Central Repository (BBI) must be used. All tubes should be labeled with the labels specific for this ancillary study. Each of these labels will have a separate sequence number (last 3 digits of number on label). The Tubes should be labeled with the HALT-C trial ID, the study visit number, and sequence numbers 305-319 as detailed on the next page.

MGH, UMASS + MCV only:

Material	Purpose	Vol (ml)	Seq #
Serum	Fibrosis	0.5	305
Serum	Fibrosis	0.5	306
Serum	Fibrosis	0.5	307
Serum	Fibrosis	0.5	308
Serum	Fibrosis	0.5	309
Serum	Fibrosis	0.5	310
Serum	Fibrosis	0.5	311
Serum	Fibrosis	0.5	312
Serum	Fibrosis	0.5	313
Serum	Fibrosis	0.5	314
Serum	Fibrosis	1.0	315
Serum	Fibrosis	1.0	316
Serum	Fibrosis	1.0	317
Serum	Fibrosis	1.0	318
Serum	Fibrosis	remain	319

4. The Serum Fibrosis AS Aliquot form (Form #102) must be completed at each visit. This form should be data entered prior to shipping these specimens to the Repository.
5. Specimens should be stored locally at –80°C. Following data entry of the Aliquot form (Form #102) these specimens will be included in the list of specimens stored at your center for shipment to the Repository. These specimens will be shipped and tracked using the Main Trial specimen tracking system.
6. These specimens should be included in the standard shipments of frozen specimens to the repository.
7. UMICHIGAN only: Serum should be separated into 20 tubes with 1.0 ml. Form #102 should NOT be completed as these specimens will not be shipped to the Repository. Instead, Form #503, the specimen collection log will be completed and faxed to NERI (617-926-0144) to be data entered. Serum will be stored at –80°C locally until sent to the testing lab. These tubes should be labeled with the HALT-C Trial ID, study visit number, and sequence numbers 680 – 699 as below:

Material	Purpose	Vol (ml)	Seq #
Serum	Fibrosis	1.0	680
Serum	Fibrosis	1.0	681
Serum	Fibrosis	1.0	682
Serum	Fibrosis	1.0	683
Serum	Fibrosis	1.0	684
Serum	Fibrosis	1.0	685
Serum	Fibrosis	1.0	686
Serum	Fibrosis	1.0	687
Serum	Fibrosis	1.0	688
Serum	Fibrosis	1.0	689

Material	Purpose	Vol (ml)	Seq #
Serum	Fibrosis	1.0	690
Serum	Fibrosis	1.0	691
Serum	Fibrosis	1.0	692
Serum	Fibrosis	1.0	693
Serum	Fibrosis	1.0	694
Serum	Fibrosis	1.0	695
Serum	Fibrosis	1.0	696
Serum	Fibrosis	1.0	697
Serum	Fibrosis	1.0	698
Serum	Fibrosis	1.0	699

4. Specimen testing and reporting

   At University of Michigan, serum PIIINP, YKL-40, TIMP-1 (refer to Amendment # 1 and 2), and HA will be run in batches following completion of enrollment and periodically thereafter. Assay results will be entered into the HALT-C Trial Data Management System at the University of Michigan (Form #103 YKL-40, Form #104 PIIINP, Form #105 TIMP-1).

Serum TGF-β1: UMICH Only

1. Inclusion/exclusion:

   Blood and liver tissue samples from **135 UMICH** patients.
   - 45 week 20 (W20) responders followed through week 72 (W72)
   - 90 randomized non-responders

2. Visit schedule

   a. Serum
   90 randomized non-responders
   Baseline (W00), week 24 (W24), month 12 (M12), month 24 (M24), month 36 (M36), and month 48 (M48)

   45 Week 20 (W20) responders (n= 180 total)
   Baseline (W00), week 24 (W24), week 48 (W48), week 72 (W72)
   45 lead-in responders

   b. Liver
   90 randomized non-responders
   Screening (S00), month 24(M24), month 48(M48)

   45 Week 20 (W20) Responders
   Screening (S00), (if available)

3. Specimen Collection and Processing (TGF-β1 UMICH)

   a. Serum
      1. 5 ml of whole blood in a Red topped tube at 6 study visits
      2. Tubes should be Centrifuged (3000 rpm X 15 min) to separate serum
      3. Aliquot serum into 8 X 1.0 ml aliquots.
      4. The Serum Fibrosis Aliquot Form (Form #102) **does not** need to be completed for these specimens as they will not be shipped to the Repository. The specimen collection will be recorded on the specimen collection log (Form #503) and faxed to NERI (617-926-0144) for data entry. The labels supplied by BBI should not be used for these specimens. The tubes should be labeled with the HALT-C Trial ID, study visit number, and sequence numbers 700 – 707 as below:

Material	Purpose	Vol (ml)	Seq #
Serum	TGF-B1	1.0	700
Serum	TGF-B1	1.0	701
Serum	TGF-B1	1.0	702
Serum	TGF-B1	1.0	703
Serum	TGF-B1	1.0	704
Serum	TGF-B1	1.0	705
Serum	TGF-B1	1.0	706
Serum	TGF-B1	1.0	707

5. Specimens stored in University of Michigan freezer at -80^{0}C
6. Samples assayed in batches after completion of enrollment, month 24 (M24) and month 48 (M48) liver biopsy.

C. Hepatic TGF-β mRNA expression: UMich Only

Quantitative RT-PCR will be used to measure TGF-β1mRNA in liver tissues obtained at S00, M24, and M48 in 80 patients at University of Michigan using the Perkin Elmer TaqMan LS-50B PCR detection system. This technique has a lower limit of detection 200 copies/ ml.

1. Inclusion/ exclusion:

   90 randomized non-responders

2. Visit Schedule

   90 randomized non-responders will provide liver tissue at screening (S00), month 24 (M24), and month 48 (M48).

3. Specimen Collection + Processing:

   a. Fresh Liver Tissue
      1. The physician performing the biopsy should obtain a liver core measuring 36mm
      2. A 3mm liver section should be cut using a scalpel or sterile razor blade.
      3. Place 3mm liver section onto a 1x1 inch square of glassine paper. This will prevent liver tissue from freezing to the side of storage tube and fingerprint contamination.
      4. Paper will curl around tissue as it is pushed into storage tube.
      5. The storage tube will be labeled with the patient name, ID number and date of biopsy. Drop storage tube carefully, using long nose tweezers, into a thermos with approximately 50cc of liquid nitrogen.
      6. Sample will freeze to – 80° C immediately.
      7. Remove storage tube with long nose tweezers, secure lid on tube and place upright in a
      8. -80° C freezer until sent to the testing lab.
      9. Collection of this specimen should be recorded on the Specimen Collection Form (#14) (refer Section E of the MOO) .Tubes should be labeled with the HALT-C Trial ID, study visit number, and sequence number as below:

Material	**Purpose**	**Vol (cm)**	Seq #
Liver	Hepatic TGF-B		708

NOTE: Liver tissue must be immediately frozen to maintain integrity of mRNA

Supplies:

- Thermos
- Thermal Gloves
- Long Nose Tweezers
- Serum Storage Tubes
- Glassine Paper

- Labels for Tubes – Which can withstand –80° C temperatures
- Sterile Scalpel or Razor Blade
- Liquid Nitrogen
- Small ruler for measuring liver tissue

10. Liver tissue will be assayed for TGF-β1mRNA content in batches at the end of the lead in phase and following the month 24 (M24) and month 48 (M48) liver biopsies. Total RNA will be determined from homogenized liver using previously described methods (14).

b. Specimen tracking

Form # 14 will be completed and entered into the data management system for each serum TGF-β1 sample obtained at University of Michigan.

c. Specimen testing and reporting

Serum TGF-B1 protein levels and hepatic TGF-B1 mRNA will be assayed at University of Michigan in batches following the completion of enrollment, month 24 (M24) and month (M48) liver biopsy. TGF-β1 protein levels and hepatic TGF-B1 mRNA will be assayed at University of Michigan in batches following the completion of enrollment, month 24 (M24) and month (M48) liver biopsy

Data will be entered into the HALT-C Data Management System at University of Michigan using Form #100 serum TGF-β1 and Form # 101 hepatic TGF-β1 mRNA.

V. Sample size

We anticipate that screening (S00) liver biopsies will be equally distributed in the 4 IF scores (3-6). The QHC values are anticipated to vary from 5% to 40%. In order to determine if there is a correlation between the serum markers and the QHC scores of at least 0.3 with 80% power at a significance level of 0.05 (two tailed), the number of patients required is 85 total. We expect that 85% or 272 of the enrolled patients at the 4 centers will have adequate liver biopsy samples. Thus we should be able to determine if the serum markers individually or in combination can predict the extent of hepatic fibrosis.

Our secondary goal is to determine if serum markers of fibrosis can be used to monitor response to IFN treatment. We anticipate that an equal proportion of the 320 patients at the 4 participating centers will be randomized to long-term IFN therapy (n=160) and no treatment (n=160). Although there are no published data to guide our estimates of the effect of long term IFN therapy on QHC in IFN non-responders, we will assume that the untreated control group will have a mean increase in QHC scores of 20% at year 4 compared to year 0 (e.g. from 30% to 36%) and the treated patients will have a mean decrease in QHC scores of 10% at year 4 compared to year 0 (e.g. from 30% to 27%). In order to detect a significant difference of 3% between the two groups with 80% power using a two-tailed test with a significance level of 0.05, the number of patients required in each group is 108 or 216 total. Assuming that the attrition rate will be 5% per year during the 4 year study and that 85% of subjects completing the trial will have adequate liver samples for QHC measurements, then 218 of the 320 patients enrolled at the 4 participating centers will have evaluable data for the longitudinal analysis.

VI. Data analysis

The mean results of the serum fibrosis marker assays will be entered into the HALT-C Trial Data Management System at University of Michigan. For the cross sectional analysis, each serum

fibrosis marker will be correlated with the continuous variable of QHC score using Pearson rank correlation methods. The serum fibrosis markers will also be correlated with the semi-quantitative ordinal values of IF and IN subscale scores using Kendall's Tau methods. In addition, correlations of the following demographic, clinical, and virologic parameters with each serum fibrosis marker will be undertaken using multiple regression methods: age, gender, life-time alcohol consumption, serum ALT level, HCV-RNA level, HCV genotype and quasispecies diversity, and hepatic iron score.

For the longitudinal cohort study, a repeated measure analysis of variance will be used to determine if significant differences exist between the QHC scores in the treated and untreated control groups. In addition, we propose to determine the integrated serum fibrosis marker/ body weight ratio as a dynamic measure of hepatic fibrosis. The AUC of each serum fibrosis marker will be calculated using the trapezoid rule (15). The AUC of each serum fibrosis marker will be correlated with the change in QHC score in each treatment group from year 0 to 2 , year 2 to 4, and year 0 to 4 using Pearson correlation method. The AUC of each serum marker will also be correlated with the IF and IN scores using Kendall's Tau method. The following parameters will also be used to predict AUC of each serum marker using multiple regression methods: Age, sex, lifetime alcohol consumption, prior IFN exposure, pretreatment HCV genotype, HCV RNA level, HCV quasispecies diversity, baseline QHC, and serum transferrin saturation. In addition, the change in HCV RNA level, serum ALT level, and HCV quasispecies diversity will be correlated with the AUC of each serum fibrosis markers using Pearson rank correlation.

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Amendment: Hyaluronic acid levels in HALT-C patients
HALT-C Serum Fibrosis Marker Ancillary Study
August 7, 2002

Principal Investigator:: Robert J. Fontana, MD (University of Michigan)
Co- Principal Investigator:: Grace Su, MD (University of Michigan)
Collaborators: Wako Pure Chemicals, Ltd, Osaka, Japan
Wako Chemicals USA, Richmond, Virginia

Relationship to HALT-C

The ancillary study "Serum Fibrosis markers in chronic hepatitis C" was reviewed and approved by the ancillary and steering committees of the HALT-C trial study group in 1999. In that proposal, I proposed to analyze serum hylauronic acid (HA), PIIINP, and laminin levels in all HALT-C patients enrolled at 4 participating centers (UMass, UMich, MCV, and MGH). The proposed time points for sample collection were week 0, week 24, month 12, month 24, month 36, and month 48 for randomized patients and weeks 0, week 24, week 48, and week 72 for responders. This study is currently approved and samples from over 500 patients have been collected and shipped to BBI for storage. Our plan is to do the serum marker assays at Umich in Dr. Grace Su's research lab. The plans for data analysis are to develop a combination marker index that will predict baseline liver histology as well as changes in hepatic fibrosis over time.

In 2001, Dr. Richard Sterling of MCV proposed an ancillary study entitled "Clinical utilities of AFP-L3 determination in early recognition, diagnosis, and prognosis of HCC patients with chronic hepatitis C". This study was approved by the ancillary studies committee and Steering Committee pending finalization of a CRADA with the external sponsor Wako Chemicals, Ltd. Per the study protocol, an aliquot of serum collected at week 0, week 24, and every 3 months thereafter in randomized patients through week 48 would be tested for AFP/ AFP-L3 (Total 16 tests/ patient) on the LiBasys autoanalyzer in Richmond, VA. In addition, serum collected at week 0, week 24, week 48 and week 72 in virological responders would be tested for AFP/ AFPL3. Per Dr. Sterling's proposal, all test procedures including the LIBAsys analyzer, chemicals, and reagents, controls, and calibrators as well as shipping costs, repository costs, and data analysis costs will be provided by Wako Pure Chemicals.

In November 2001, I met with officials from Wako and learned that the autoanalyzer that would be used for the AFP-L3 testing can also analyze HA levels. Pilot testing with banked serum from Dr. Fontana's research lab has taken place demonstrating the feasibility of HA testing from patient sera (see below). The following proposal was developed to provide serum HA testing in all of the patients enrolled at all participating sites in the HALT-C trial according to the same schedule and timepoints as outlined in Dr. Sterling's proposal.

Background

The natural history and long term prognosis in chronic hepatitis C is closely linked to the development of hepatic fibrosis (1,2). Recent studies suggest that IFN may have anti-fibrotic and anti-inflammatory effects in addition to its antiviral effects (3). However, conclusive data are lacking due to the small size of the studies, short duration of treatment and follow-up, and difficulties in quantifying hepatic fibrosis. Liver biopsy is frequently considered the best established means of assessing disease activity and severity in chronic hepatitis C. However, liver biopsy is expensive and associated with a small but definite risk of complications. In addition, substantial sampling error may occur as well as interobserver variability in interpretation (4,5). As a result, sensitive and reliable serological assays for the non-invasive detection and monitoring of hepatic fibrosis are needed.

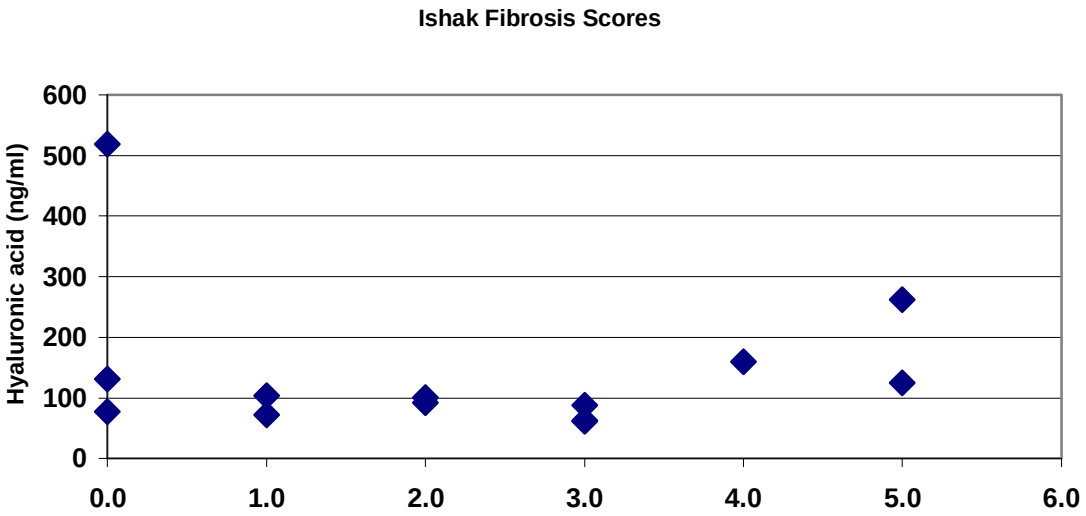
Hyaluronic acid (HA) is a high molecular weight linear polysaccharide that is ubiquotously distributed in the extracellular spaces of the body(6). In the liver, HA is synthesized by stellate cells and degraded by sinusoidial endothelial cells (7). Since serum HA levels are known to be increased in patients with advanced hepatic fibrosis, serum HA levels have been proposed as a non-invasive marker of fibrosis and cirrhosis (8,9). In one large study of 486 hepatitis C patients, a serum HA level of < 60 ug/ l had a NPV for fibrosis of 93% and a NPV for cirrhosis of 99% (8). In two longitudinal studies of IFN therapy, HA levels declined significantly when hepatic fibrosis improved, increased significantly when fibrosis worsened, and remained unchanged in subjects with stable hepatic fibrosis (10,11). These studies suggest that serum HA levels may be a useful marker of hepatic fibrosis in hepatitis C and in monitoring fibrosis progression over time.

Pilot data

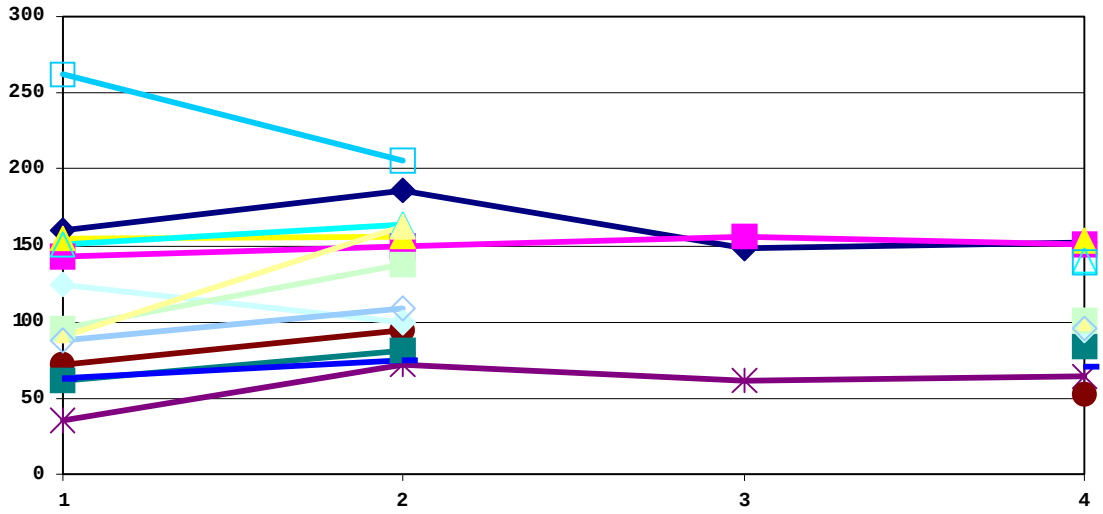
Frozen serum samples from 21 hepatitis C patients and 4 healthy controls stored in Dr. Fontana's research laboratory were assayed on the LIBAsys analyzer at Wako Laboratories in May 2002 and repeated in July 2002.

Patient Demographics: There were 14 male and 7 female hepatitis C patients whose mean age was 42 years (range: 18 to 51). There was also 2 male and 2 female healthy controls with a mean age of 34 years (range 24 to 39). There was no apparent relationship between subject age and HA level or subject gender and HA level.

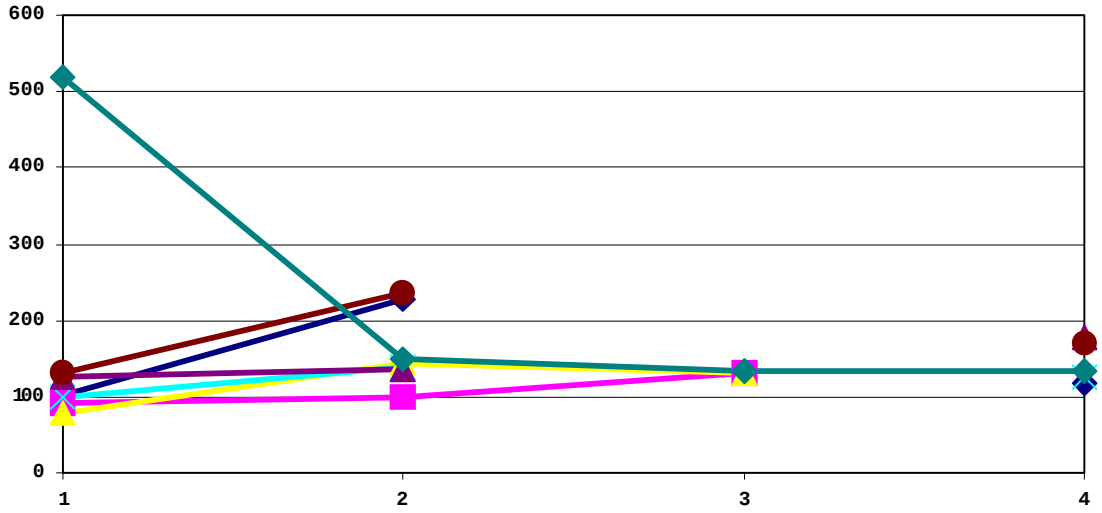
Liver histology: Baseline liver biopsies that had been scored for hepatic fibrosis using the ISHAK scoring system were available in 12 hepatitis C patients. As seen in the attached graph, there appears to be a correlation between Ishak fibrosis score and HA level if the single patient with an IF score of 0 and HA level of 522 is excluded. This patient may be an outlier due to baseline hypertriglyceridemia (334 mg/dl) and collection of his baseline sample immediately after eating.



IFN and Ribavirin therapy: Stored serum were available in 13 patients who had received IFN and ribavirin in a prospective clinical trial conducted between '96 and '98. The patients included 4 with a SVR, 7 non-responders, and 2 patients with virological relapse during follow-up. As seen below there was no discernible trend in HA values over time. Of note these specimens were not uniformly collected in fasted patients. (Time point 1= pretreatment, Time point 2 & 3 = on treatment Time point 4 = Post-treatment follow-up)

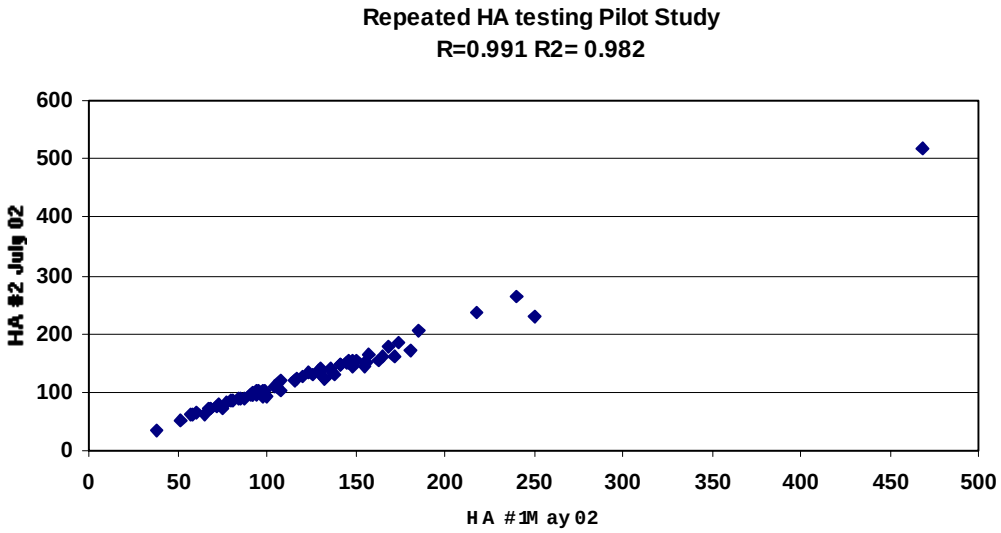


PEGIFNα2a and ribavirin therapy. A limited number of stored sera were available in 8 patients receiving PEGIFNα2a and ribavirin in an ongoing clinical trial. As seen below, there was no discernible change in HA levels over time with antiviral therapy. (Time point 1= pretreatment, Time point 2 & 3 = on treatment Time point 4 = Post-treatment follow-up)



Fasting vs feeding: Previous studies have demonstrating that eating can transiently increase serum HA levels due to increased hepatic lymph flow from visceral organs (12,13). To test this phenomenon, 4 healthy young volunteers without known liver disease had blood drawn in a fasting state and then within 1 hour of eating breakfast. Eating led to a mean increase in HA levels of 18%.

Coefficient of variation. All of the samples sent to WAKO labs in May 02 were retested in July 02 to determine the effect of freezing and thawing on test results and the coefficient of variation. Overall, the repeat testing of HA was a mean of 2.3% lower than the 1st test. The correlation between test #1 and #2 was excellent with R= 0.991.



Hypotheses and Aims

The aim of this ancillary study is to evaluate the utility of fasting serum HA levels in predicting hepatic fibrosis in chronic hepatitis C. In addition, we will determine if changes in HA levels during the course of the HALT-C trial correlate with changes in hepatic fibrosis on liver biopsy in patients receiving long term PEG IFN and untreated controls. The hypotheses to be tested include:

1). Serum levels of HA correlate well with Ishak fibrosis staging and computerized morphometry in CHC patients

2). Changes in serum levels of HA correlate with changes in hepatic fibrosis in patients entering the randomized phase of HALT-C.

3). Long term PEG IFN induces significant reduction in hepatic fibrosis as well as serum HA levels even in the absence of a virologic response

4). Changes in serum HA levels correlate with virological response (i.e. sustained, relapse, breakthrough) in patients entering the responser arm of the HALT-C trial

Study Design

HALT-C patients enrolled at all 10 participating sites have serum collected, aliquoted and shipped to BBI for storage. We propose that samples that will be pulled for AFP/ AFP-L3 testing in randomized and week 20 responders be assayed for HA levels upon shipment to Wako Labs.

<u>HA assay</u>

HA is a linear polysaccharide built from a dissaccharide unit consisting of D-glucuronic acid and D-N-acetylgluosamine. The Wako LiBA HA assay is an in vitro assay for the quantitative determination of HA levels in serum. The assay method uses a liquid-phase binding reaction between antigen (HA) and antibody (HABP antibody). Bound and free forms of the antigen/ antibody complex are separated by column chromatography without the need for a solid phase. LBA HA can offer fully automatic and highly precise measurement of HA by using an automated immunological analyzer.

Since the HA analyzer is physically coupled with the AFP/ AFP L3 analyzer, a 20 ul aliquot of serum can be used to determine HA levels. Samples will be analyzed once. Specimens will be retested on a random basis to determine if there is any degradation of sample integrity on a schedule determined by NERI. In addition, WAKO will perform in-house validation studies every 6 months to insure test accuracy and provide those test results to NERI. WAKO will provide NERI with adequate information on quality control and temporary storage of samples. The results from this study will not be applied to direct patient care.

Assay Performance characteristics
Expected (normal) values < 50 ng/ ml (Japanese patients)
Lower detection limit. When a sample with HA concentration of 0 ng/ ml is assayed 3 times in a run, the mean value + 2sd is less than 10 ng/ ml.
Accuracy- When a control serum with known concentration of HA is assayed, a measured value is within 15% of known concentration
Measurable range: 10 to 1000 ng/ ml
Precision: When a sample is assayed 5 times in a run, the coefficient of variation is within 15%.

Schedule of testing
Testing on stored frozen serum will take place at Wako Labs in Richmond VA. Coded aliquots of 120 ul of serum will be shipped at regular intervals from BBI to Wako Labs. Testing for HA levels will be performed on baseline (W00), Week 24, and every 3 months during the randomized phase for non-responders in the HALT-C trial (Total of 16 tests/ patient over 48 months). For patients with a virological response during the lead-in phase, specimens collected at W00, W24, Wk 48, and wk 72 will be tested (4 tests/ patient). Anticipating that there will be 900 randomized patients and 400 week 20 responders, the projected number of specimens to be assayed is 900 x 16 + 400 x 4 = 16,000 assays.

Data Analysis
For the cross-sectional analysis, HA levels at week 0 will be correlated with the continuous variable of hepatic fibrosis determined by computerized morphometry (Dr. Zak Goodman) using Pearson rank correlation methods. The serum fibrosis markers will also be correlated with the semi-quantitative ordinal values of the Ishak Fibrosis and Ishak inflammatory subscale scores using Kendall's Tau methods. In addition, correlations of other important demographic, clinical, and virological data will be undertaken using multiple regression methods.

Changes in hepatic fibrosis occur slowly over time. Therefore, although HA data will be available from patients at 3 month intervals, we propose to use the Week 0, week 24, month 12, month 24, month 36, and month 48 HA results for analysis in the randomized patients in HALT-C. A repeated measure analysis of variance will be used to determine if significant differences exist between the computerized morphometry scores in the treated and untreated control groups. In addition, we will determine the integrated serum fibrosis marker/ body weight ratio as a dynamic measure of hepatic fibrosis using the AUC of HA calculated using the trapezoid rule.

The HA data in patients in whom other serum markers are being tested in the currently funded ancillary study will be used to develop a combination marker index to predict baseline liver histology and changes in hepatic fibrosis over time.

Budget
All HA test procedures including the LiBAsys analyzer, chemicals, reagents, controls, and calibrators as well as shipping costs will be provided by Wako Pure Chemicals. In addition, a small % effort for the PI and data coordinatoring center will be provided by Wako. No additional HALT-C funds will be required for this proposal (See attached budget). The specimens will be batched and sent from each participating site to BBI every 3 months. Specimens will be shipped from BBI to WAKO at Biosafety level 2 on dry ice using Federal Express with no more than 4000 ml of serum shipped in each container. Unused serum will be shipped back to BBI for permanent storage. Note all other shipping, repository, and analytical costs are presumed to be covered in full by the AFP-L3 budget submitted by Dr. Sterling.

Problems and pitfalls
1. Fasting vs fed - Published reports as well as our own pilot data suggest that feeding may lead to substantial increases in serum HA levels due to increased splanchnic lymph flow. As a result, it is

imperative that all test results that will be used for analytical purposes be obtained in the AM from fasted subjects

2. Healthy controls- The normal values for healthy adults without liver disease provided by WAKO were obtained in healthy Japanese individuals. However, the results from our pilot testing suggest that the normal values for US healthy controls may be substantially higher (100 ng/ml vs 50 ng/ml). A prospective study that will assess normal values in a large sample of male and female healthy adult controls will be conducted by Dr. Fontana with WAKO as a separate project. This data will be provided to the HALT-C study group and data coordinating center.

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Amendment #1 YKL-40
HALT-C Serum Fibrosis Marker Ancillary Study
February 19, 2003

Background

At the time of approval of the serum fibrosis marker ancillary study in '00, the selection of serum markers was based upon the available medical literature and serum PIIINP, HA, and laminin were proposed. Since that time published data has demonstrated that serum YKL-40 may prove to be a useful and complementary marker for assessing hepatic fibrosis and change over time in hepatitis C patients (1,2). Therefore, we propose to measure serum YKL-40 levels at the previously determined study visits using collected samples in the HALT-C serum fibrosis marker ancillary study.

YKL-40 also known as human glycoprotein 39 or CHONDREX is a 40 kd glycoprotein secreted by chondrocytes, synovial cells, activated macrophages, and neutrophils (3). The function of YKL-40 is unknown but it is believed to play a role in tissue remodeling and degradation of extracellular matrix due to its pattern of expression (4). The molecule is a lectin that has both heparin and chitin binding domains (4). Recently, cross-sectional studies have suggested that YKL-40 may be a useful marker of hepatic fibrosis in patients with alcoholic liver disease and viral hepatitis (5, 6,1). The serum level of YKL-40 has been shown to significantly correlate with the degree of hepatic fibrosis (5,6). In 103 patients with hepatitis C, YKL-40 levels demonstrated a stronger correlation with the degree of hepatic fibrosis (r= 0.618) than serum PIIINP (r=0.395) and hyaluronic acid levels (r=0.458) (1). YKL-40 levels also correlated with the amount of hepatic fibrosis assessed by computerized morphometry using Sirius red staining (1). These studies suggest that YKL-40 is an independent marker of liver fibrosis in hepatitis C patients and may be superior or complementary to PIIINP and HA in assessing hepatic fibrosis.

To date there are 2 published studies assessing the utility of serial YKL-40 levels in patients with chronic liver disease (7,2). Nojgaard et al reported on the utility of baseline YKL-40 and PIIINP levels in predicting survival in 370 European patients with alcoholic liver disease (7). In this study, both serum YKL-40 and PIIINP levels at baseline strongly correlated with liver histology at enrollment. During a median follow-up of 1.5 years, patients with a normal YKL-40 had significantly improved survival compared to patients with an elevated level. However, in a multiple regression model, baseline serum YKL-40 level was not an independent predictor of outcome.

Afdhal et al have reported on the use of baseline and serial YKL-40 levels in Egyptian patients with acute HCV alone (n=20) and acute HCV with schistosomiasis (n=22) (2). During this unique natural history study wherein patients underwent a baseline and follow-up liver biopsy at 8 years, the rate of Ishak fibrosis progression was markedly greater in schistosomiais patients (0.61 units/ yr) compared to HCV alone patients (0.14 units/ yr). Serum YKL-40 levels paralleled the fibrosis progression rates in both groups (r=0.892). In particular, serum YKL-40 levels increased sharply in patients with Ishak fibrosis $\geq$ 3. YKL-40 levels showed a linear correlation with TGF-B levels (r=0.897) and hepatic mRNA levels of YKL-40 and TGF-B correlated with serum levels. A ROC curve analysis revealed that YKL-40 was superior to PIIINP in monitoring fibrosis progression (AUC for YKL-40 0.873 $\pm$ 0.7 vs. PIIINP 0.59 $\pm$ 1.3) and in discriminating between patients without fibrosis and those with fibrosis (AUC 0.889 $\pm$ 0.03, vs PIIINP 0.644 $\pm$ 1.42) This unique longitudinal study demonstrates that serum YKL-40 levels can accurately determine the rate of fibrosis progression in hepatitis C patients.

Angiographic studies assessing YKL-40 levels from arterial and hepatic vein samples demonstrate a significant concentration gradient across the liver suggesting that YKL-40 is released from the liver or splanchnic circulation (8).

Immunohistochemical staining of liver tissue demonstrated positive staining for YKL-40 antigen in areas with fibrosis and particulary in areas with active fibrogenesis (5). However, YKL-40 staining has

not been seen in hepatocytes or in normal liver tissue. Additional studies from Nid Afdhal using in-situ hybridization have demonstrated YKL-40 message in edges of liver tissue with active fibrogenesis (unpublished observations).

YKL-40 assay

Because it uses an ELISA format, the YKL-40 assay is simple to perform. Each test kit contains 96 wells wherein 40 independent samples can be run in duplicate along with standards. Each assay requires 20 ul of serum. YKL-40 reference ranges have been established for healthy adult males (n=103) and healthy adult females (n=226) age 18 to 60 (9). The lower limit of detection for the assay is 20 ng/ml. The within run coefficient of variation is 5.6 to 6.6% and the between run coefficient of variation is 6.0 to 7.0%. Repetitive freezing and thawing has no significant effect on measured YKL-40 concentrations. However, the time at which a blood sample is allowed to clot at room temperature before it is centrifuged can effect assay results (10)

In the HALT-C trial, we propose that an adequate number of YKL-40 test kits be provided by the manufacturer (Quidel Corporation, San Diego CA) to Dr's. Fontana and Su at the University of Michigan. Each sample will be run in duplicate. Initial testing will focus on W00 samples and then batches of samples will be run on responder patients and randomized patients during years 5 through 9 of HALT-C.

Using the projected number of patients enrolled at UMich, MGH, MCV and UMass as of January 31, 2003, the total number of samples to be tested is as follows:

	W00	**W24**	**W48**	**W72**	**Mon 12**	**Mon 24**	**Mon 36**	**Mon 48**	**Total**
MGH	69	69	14	14	55	55	55	55	386
UMASS	111	111	47	47	81	81	81	81	640
MCV	221	221	66	66	187	187	187	187	1322
UMich	110	110	39	39	103	103	103	103	710
total	511	511	166	166	426	426	426	426	3058

It is anticipated that a minimum of 80 YKL-40 kits will be required to complete this project.

Data analysis

The serum YKL-40 data will be entered into the HALT-C database (modification of form #100). Correlations between YKL-40, PIIINP, laminin, and HA levels with baseline, year 2, and year 4 histology will be undertaken as previously described. In addition, longitudinal data will be analyzed as previously described.

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Amendment #2 TIMP-1
HALT-C Serum Fibrosis Marker Ancillary Study
February 16, 2004

Background

At the time of approval of the serum fibrosis marker ancillary study in '00, serum PIIINP, hyaluronic acid, and laminin were proposed as the marker panel to test. Since that time, serum YKL-40 has been added to the panel in light of its association with hepatic fibrosis (Amendment #1, February 2003). Upon careful review of additional publications on hepatic fibrosis in chronic hepatitis C (CHC), it appears that serum TIMP-1 may be superior to serum laminin in detecting baseline hepatic fibrosis as well as changes in fibrosis over time (1,2, 3). For example, the 3 markers with the greatest sensitivity and specificity for detecting advanced fibrosis from the EUROGOLF study of 1,000 patients are TIMP-1, HA, and PIIINP (3). Similarly, TIMP-1 is a core component of the PROMETHEUS FibroSpect algorithm for predicting hepatic fibrosis in CHC along with hyaluronic acid and alpha2-macroglobulin (4). Lastly, one study of 31 Italian CHC patients treated with Interferon and followed up for 2 years demonstrated no changes in serum laminin levels either during or after treatment (5). Therefore, I propose to measure serum TIMP-1 levels in lieu of serum laminin levels at the previously determined study visits in HALT-C for the following reasons

1. TIMP-1 levels correlate well with hepatic fibrosis in CHC patients
2. The limited laminin assay dynamic range of 0 to 4 u/L makes it a insensitive assay for detecting changes in hepatic fibrosis over time
3. The TIMP-1 assay has a large dynamic range of 0 to 200 ug/l allowing for greater detection of hepatic fibrosis changes over time
4. A commercially available sandwich ELISA kit for human TIMP-1 is available (Quantikine, R & D systems).

Hepatic fibrosis from chronic necroinflammatory liver disease is characterized by a shift in the extracellular matrix from the normal low density matrix consisting of Type IV collagen and elastin to a high density matrix consisting of Type 1 and 3 collagens (6). Matrix metalloproteinases (MMP'S) are a family of zinc-dependent endopeptidases produced in the liver by stellate cells that degrade extracelullar matrix during both liver injury and repair. The activity of MMP's is controlled by regulation of expression and secretion, by proteolytic activation of pro-enzymes, and by the Tissue inhibitors of MetalloProteinases (TIMPs). MMP's are secreted in the inactive proenzyme (pro MMP) and activated in the ECM. MMP-2 helps degrade the low density ECM during active fibrogenesis while MMP-1 helps degrade the high density ECM during fibrolysis and tissue remodeling. TIMP's form 1:1, non-covalent complexes with MMP's in general but not with any particular MMP. TIMP-1 is an inducible protein that is widely synthesized by many cells and tissues while TIMP-2 is constitutive. Transcription of the TIMP-1 gene is induced by proinflammatory cytokines and TGFB1 that enhance fibrogenesis brolysis.

TIMP-1 is a 184 amino-acid residue glycosylated protein whose N-terminal domain contains sites that bind to the MMP substrate binding site. The TIMP-1/ MMP complex can disassociate to yield enzyme and active TIMP-1. High levels of circulating TIMP-1 indicate inhibition of MMP's that degrade collagen (i.e. fibrolysis) and are associated with enhanced fibrogenesis. The TIMP-1 assay measures both free TIMP-1 and TIMP-1 that is complexed with MMP in the circulation (7).

In one study of 59 non-cirrhotic CHC patients, 19 cirrhotic CHC patients, and 30 healthy controls, plasma TIMP-1 levels were determined using the Quantikine assay (2). Significant differences were noted between healthy controls and CHC patients with no fibrosis, CHC patients with Ishak 1 to 4, and CHC patients with Ishak fibrosis of 5-6. In addition, quantification of TIMP-1 mRNA was also determined in the 59 non-cirrhotic CHC patients. The liver TIMP-1 mRNA levels significantly correlated with plasma TIMP-1 levels (r= 0.54) and the degree of hepatic fibrosis (r=0.46).

In another study, YKL-40, MMP-2, PIIINP, and TIMP-1 levels were measured before, during and after Interferon and ribavirin therapy in 51 CHC patients who had previously failed IFN therapy (1). Six months after treatment, the 30 sustained responders had significantly lower TIMP-1 levels compared to baseline (106 ug/L vs 160 ug/L, $p < 0.001$). In addition, the 19 virological non-responders also tended to have a decrease in TIMP-1 levels following treatment compared to baseline but to a lesser extent (130 ug/L vs 157 ug/L, $p < 0.05$). Unfortunately, liver biopsies were not obtained in all subjects prior to treatment and none of the patients following therapy.

TIMP-1 assay

The Quantikine (R & D Systems, Minneapolis, MN) TIMP-1 immunoassay is a solid phase ELISA kit designed to measure total TIMP-1 concentrations in serum and plasma. It contains NSO-expressed recombinant human TIMP-1 and antibodies raised against the recombinant protein. The assay employs a quantitative sandwich ELISA technique.

The lower limit of detection is 0.08 ug/l and the upper limit is 200 ug/L. Per the package insert, the inter-assay CV is 4-5% and the intra-assay CV is 4-5%. The range of TIMP-1 in serum reported by the manufacturer from 60 healthy people is 87-524 ug/l with a mean of 190 ug/L. In CHC patients, the values of TIMP-1 in plasma and serum range from 50 to 900 ug/L with normal controls having a mean of 71 ug/l.

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Amendment #3
HALT-C Serum Fibrosis Marker Ancillary Study
Approved: December 10, 2004

Background
Per a previously executed Clinical Trial Agreement with NIDDK, WAKO laboratories of Richmond, VA has agreed to assay HALT-C serum samples from patients enrolled at all sites for hyaluronic acid, total serum AFP and AFP-L3 as part of the approved Serum Fibrosis Marker and HCC ancillary studies (Fontana and Sterling, respectively). WAKO has completed analysis of most of the Wk 00, Wk 24, and Wk 48 samples using only ~ 40 ul of the sample in April 2004 and has the remaining serum sample in their freezer. They will proceed with testing of additional samples moving forward.

WAKO laboratories has indicated they are willing to perform testing for additional analytes of interest from these sera at no additional cost to HALT-C (Pending amendment to the Clinical Trial Agreement). WAKO can detect multiple analytes using anywhere from 3 to 20 ul of serum/ assay.

A list of tests that would be of interest to the HALT-C study group has been composed. Due to prior freezing and thawing, meaningful data can not be obtained for HDL cholesterol, total cholesterol, and Apolipoprotein A1 from previously thawed samples. However, serum total bile acids, GGTP, total bilirubin, and rheumatoid factor could be assayed from all or some of the previously shipped and tested samples that are being assayed already for HA and AFP-L3. In addition, moving forward HDL and total cholesterol assays could be run on freshly frozen sera that are initially defrosted for HA and AFP/ AFPL3 testing

Proposal
For serum samples that are already being shipped to WAKO for hyaluronic acid and AFP-L3 testing, we propose that these sera also be analyzed for the following analytes from week 0, 24, 48, 72 and every 6 months in the randomized phase. These additional assays will likely require at least 80 ul of serum and possibly more if duplicate testing is required. In the event that there is inadequate sera available, the assays will be performed in the following priority scheme:

1. Direct Bilirubin- Serum total bilirubin levels are a well recognized marker of liver disease severity and an integral component of the CTP score which is being used in the HALT-C trial for determination of clinical endpoints. We propose to analyze Wk 0, wk 24, Wk 48, Wk 72, and q 6 month samples from the randomized phase for direct bilirubin.
    a. Volume = 10 ul Range: 0 to 20 mg/dl
    b. Assay issues: Bilirubin is rapidly oxidized on exposure to light. It is believed to be stable when frozen at -80° C for prolonged periods of time. No effect of feeding. If level exceeds 20 mg/dl, sample should be diluted 1:1 and repeated.
    c. Proposed data interpretation: Compare results of WAKO direct bilirubin levels to those reported by local labs. During lead-in phase, identify profile of direct bilirubin in sustained responders vs non-responders vs relapsers. In longitudinal randomized phase, compare direct bilirubin in PEG vs untreated patients.
2. Total bile acids- There is an extensive literature correlating serum total bile acids with the severity and prognosis of liver disease. Furthermore, cholate clearance is one of the metabolic tests in the approved Quantitative tests of liver function ancillary study for predicting clinical outcomes in HALT-C. Therefore, we propose that baseline, Wk 24, Wk 48, Wk 72, and q 6 month samples from the randomized phase be assayed for total serum bile acids.
    a. Volume = 20 ul Range: 0 to 150 umol/ L
    b. Assay issues: Need to run control sera from healthy volunteers (up to 6 umol/L). Provided by WAKO. Stable if frozen at -80 C for 1 year.
    c. Proposed Data analysis= Correlate Wk 0 levels with histology, portal HTN parameters, laboratory markers of disease severity. During lead-in phase, identify profile of total

bile acids in sustained responders vs non-responders vs relapsers. In longitudinal randomized phase, compare total bile acids in PEG vs untreated patients and in patients who reach a clinical outcome amongst cirrhotics or histological progression amongst non-cirrhotics.

3. GGTP- Serum GGTP level has been proposed as a sensitive marker for liver disease including alcoholic liver disease and more recently NAFLD (NHANES). In fact, GGTP is frequently assayed in hospital labs as part of a liver biochemical profile and GGTP, apolipoprotein A1, haptoglobin, α2macroglobulin, and total bilirubin are the components of the FIBROTEST for assessing hepatic fibrosis. We propose to analyze Wk 0, wk 24, Wk 48, Wk 72, and q 6 month samples from the randomized phase for GGTP
    a. Volume = 10 ul Range: 9 to 2000 IU/L
    b. Assay issues: Normal controls and samples above the upper limit to be provided by WAKO. Values interpreted with normal controls in men (12-64 IU/L) and females (9-36 IU/L)
    c. Proposed data interpretation: Correlate Wk 0 levels with histology, portal HTN parameters, laboratory markers of disease severity, lifetime alcohol consumption history, other serum fibrosis markers, NAFLD/ NASH features on liver biopsy, and insulin resistance profiles. During lead-in phase, compare GGTP in sustained responders vs non-responders vs relapsers. In longitudinal randomized phase, compare GGTP in PEG vs untreated patients and in patients who reach a clinical outcome amongst cirrhotics or histological progression amongst non-cirrhotics.
4. C-reactive protein- CRP is a protein that binds the C-Polysaccharide of the cell wall of Streptococcus pneumoniae. CRP is an acute phase reactant that rises dramatically in the case of inflammation or tissue destruction. It is associated with clinical outcomes in cardiovascular diseases.
    a. Assay volume=14 ul Range: 2.5 to 200 mg/L
    b. Assay issues: Normal values are affected by aged, gender, and diet. WAKO will need to provide normal values.
    c. Proposed data analysis: Correlate baseline CRP values with liver histology and clinical outcomes during the randomized phase of HALT-C.
5. Total Cholesterol- Total cholesterol is one of the 5 features of the metabolic syndrome and is strongly associated with the risk of cardiovascular disease.
    a. Assay volume- 6 ul Range: 2 mg/dl to 800 mg/dl
    b. Assay issues: Specimens are stable if stored at -70 C for many years. Feeding may lead to spurious increases in total cholesterol levels.
    c. Proposed data analysis: Correlate total cholesterol levels with the degree of steatosis and steatohepatitis in the baseline liver biopsy. Determine the frequency of metabolic syndrome in HALT-C patients during the longitudinal phase of the trial in PEG treated vs untreated.
6. HDL cholesterol- There is a strong inverse relationship between HDL cholesterol and cardiovascular disease. HDL cholesterol is also a component of the metabolic syndrome.
    a. Assay volume: 5 ul Range: 1 to 180 mg/dl
    b. Assay issues; Sample should be drawn from a fasting patient to avoid spurious decreases. Some drugs may interfere with the HDL assay.
    c. Proposed analysis: Correlate HDL cholesterol with baseline steatosis and steatohepatitis histological scores. Determine the frequency of metabolic syndrome during the longitudinal phase of the trial in Peg treated and untreated patients.
7. Rheumatoid factor- RF is an autoantibody of human immunoglobulin (IgG). RF presence and level is a marker of rheumatoid arthritis and other autoimmune diseases. Detectable RF levels have been reported in 59% to 81% of HCV patients of unclear clinical significance although associations with cyroglobulinemia have been suggested (Pawlotsky et al Hepatology 1994; 19: 841-848). Whether detectable RF levels prior to or during antiviral therapy in patients receiving Interferon who develop autoimmune phenomena such as immune mediated

hemolytic anemia, or other autoimmune side effects have not been prospectively studied. Therefore, we propose to measure RF levels from all HALT-C patients at Wk 0 and serial samples in patients with cryoglobulinemia or autoimmune side effects of Interferon during the lead-in or randomized phase of HALT-C.

a. Assay volume = 9 ul Range: 1 to 500 IU/L
b. Assay issues: Normal values are less than 30 IU/ml but depend on age, gender and other factors. No effect of feeding.
c. Proposed data analysis- Describe the prevalence and titer of Wk 0 RF levels in HALT-C patients. Determine if there is any relationship between Wk 0 RF levels and presence of clinically apparent cryoglobulinemia (recorded on medical history form) and other autoimmune diseases. For patients who develop immune mediated side effects from Interferon during the Lead-in and randomized phase of HALT-C, determine if there is a characteristic pattern of RF titer changes prior to and following the event (? Case control study like for HCC patients).

After completing the above, if there is adequate serum remaining then the following two additional analytes will be tested.

8. Transferrin- Transport of iron throughout the body is accomplished by the binding of iron to a molecule called transferrin. The unsaturated iron binding capacity of transferring is termed the UIBC. Elevated levels of UIBC are seen in states of iron deficiency anemia. Transferrin is an 80 kdA glycoprotein synthesized in the liver which can be quantified using a turbidimetric immunoassay.
    a. Assay volume = 3 ul Range: 220- 700 mg/dl
    b. Assay issues- Serum samples should be utilized. Levels stable if sample is immediately frozen. Samples should be collected in a fasted state.
    c. Proposed data analyses- The serum transferrin level will be correlated with serum iron stores, ferritin, and hepatic iron levels. Transferrin levels may be useful in identifying patients at risk for fibrosis progression.
9. Unsaturated iron binding capacity (UIBC)- This analyte can be measured from serum using a bathopenanthroline method.
    a. Assay volume = 10 ml Range: 191-600 ug/ dl
    b. Assay issues- Samples should be collected in a fasting state. Hemolysis may lead to spuriously high iron levels and low UIBC levels.
    c. Proposed data analyses- The UIBC will be correlated with the TIBC- iron levels and serum ferritin levels. The UIBC will be compared to hepatic iron levels and can be used in potentially identifying risk factors for HCC and fibrosis progression.

Data entry into DMS

The results of all test results will be sent to Dr. Fontana for review. Once final results have been verified, they will be entered into the DMS by NERI. All subsequent analyses will be conducted per HALT-C study protocol guidelines by NERI.

Budget

WAKO is agreeing to run the additional analytes at no additional cost to the HALT-C study group. Since the serum samples are already being pulled from the Repository by BBI for the Serum fibrosis markers and HCC ancillary studies, the costs of shipping samples has already been provided for. Data analyses will be completed by NERI. Therefore, there is no request for incremental funding to conduct the proposed work.