Iron & HFE Mutations Snap Shot

Sites Participating:
All sites participate in HFE mutational analysis, and measurement of serum iron, total iron binding capacity and serum ferritin.

Hepatic Iron Concentration patients only at University of Massachusetts (site #11), University of Connecticut (site#11), University of California-Irvine (site #15)

Principal Investigator: Herbert L. Bonkovsky, MD (University of Connecticut)

Study Name: IRON AND HFE MUTATIONS ANCILLARY STUDY

Separate Consent Form: No

Withdrawal Form: No

Eligible Patients: Lead-in, Randomized, Breakthrough/Relapser; Express
(W20 Responders are not eligible)

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

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

Lead-In Phase

Visit Number ➔	Form #	S00	W00	W20	W24
HFE Gene Testing	Dataset		X		
Histopathology	181	X			
Hepatic Iron Concentration	182	11,15			
Serum Iron	183				
Iron & HFE aliquot form[1]	184	11,15			
Liver Biopsy	14	X			

Randomized Phase

Visit Number ➔	form #	R00	R00	M12	M24	M48
		Express	B/R			
HFE Gene Testing	Dataset	X				
Histopathology	181				X	X
Hepatic Iron Concentration	182				11,15	11,15
Serum Iron	183				X	X
Iron & HFE aliquot form[1]	184				11,15	11,15
Liver Biopsy	14				X	X

[1] Form 184 was implemented for site 11 UMASS/UCONN in July 2003.

Iron & HFE Mutations Ancillary Study

Principal Investigator: Herbert L. Bonkovsky, MD

I. Purpose

To assess whether iron in the liver (or other tissues) and/or HFE gene mutations play a co-morbid role in progression of chronic hepatitis C or influence response to initial or long-term therapy or other potential co-morbid factors (especially fatty liver / obesity / insulin resistance).

II. Summary Description:

This will be an observational and correlational study that will draw upon the clinical, biochemical, and histopathological data that will be collected as part of the HALT-C Main Trial Protocol. It will also draw upon data collected as part of other Ancillary Studies, especially those on Immunology/Virology, Portal Hypertension, and the Modifiable Risk Factors.

In addition, we will perform HFE mutational analyses on all patients who enter the main trial, both the Lead-in phase (therapy with Peginterferon alfa-2a and ribavirin) and the Randomized phase (long-term therapy with Peginterferon alfa-2a vs. no treatment). Semi-quantitative assessment of hepatic iron content will be reported for all liver biopsies by the periodic consensus meetings of the Central Pathology Committee, and Barbara F. Banner, MD, will perform and report a more detailed study of stainable iron in portal tract cells for all biopsies.

A sub-set of patients, namely, those who will be recruited, biopsied, and followed at University of California, Irvine (PI: Tim Morgan, MD) and University of Connecticut / University of Massachusetts (PI: Herbert L. Bonkovsky, MD and Savant Mehta, MD), whenever possible, will also have small portions (~ 10 mg wet weight) of liver tissue set aside for measurement of hepatic iron concentrations (HIC). These measurements will be carried out in the Liver Research Labs of University of Connecticut / University of Massachusetts, where the *HFE* mutational analyses will also be performed.

III. Inclusion / Exclusion Criteria:

A. For *HFE* mutational analysis, and measurement of serum iron, total iron binding capacity and serum ferritin: All patients enrolled in the HALT-C Trial, both the Lead-in phase (treatment with Peginterferon alfa-2a and ribavirin) and the Randomized phase (long-term treatment with Peginterferon alfa-2a vs. no treatment).

B. Hepatic iron concentration: Patients at University of California, Irvine and University of Massachusetts / University of Connecticut.

C. Analysis of iron in biopsies (semi-quantitative and stainable iron in portal tract cells): All patients who enter the HALT-C Main Trial; all biopsies at Screening (S00), Year 2 (M24), and Year 4 (M48).

IV. Schedule of Visits and Specimen Collection

A. Serum iron, total iron binding capacity, and serum ferritin: additional measurements at Year 2 (M24) and Year 4 (M48): all clinical centers.

B. Biopsies for hepatic iron concentration measurements at screening, Year 2 (M24) and Year 4 (M48): University of California, Irvine and University of Connecticut / University of Massachusetts.

C. The analysis of iron in biopsies (semi-quantitative and stainable iron in portal track cells) will be done using biopsies collected for the HALT-C Main Trial protocol. No additional specimen collection is needed.

V. Specimen Collection, Handling and Shipping Instructions

A. Blood draw

Serum iron and iron binding: all study sites: At screening (S00), serum iron, total iron binding capacity and serum ferritin will be collected as part of the HALT-C Main Trial protocol, Form #4 Screening Checklist. Additional measurements will be made at Year 2 (M24) and Year 4 (M48) for patients participating in this ancillary study, Form #183 Serum Iron – Iron & HFE Gene Mutation AS. Serum iron, total iron binding capacity and serum ferritin can be measured using the blood being drawn for fasting chemistries at these times; the tests need to be ordered on the form being submitted to the local lab.

B. Liver biopsies for Hepatic Iron Concentration: for University of California, Irvine and University of Connecticut / University of Massachusetts:

At the time of each biopsy, assuming sufficient tissue is obtained, about 10 mg wet weight of liver will be placed directly onto a small piece of glassine paper, and the paper then placed into a 2 ml aliquot tube provided by the Central Repository. The vial will be labeled with the patient's I.D. number, study visit number, and the collection date, and the vial will be frozen (-20° C or less).

Complete Form #14, Specimen Collection, Section E. The size of the specimen collected should be recorded, along with the purpose (Iron AS) and how collected/handled (1-Room temp-fresh).

Specimens collected at UC Irvine, UMASS and UCONN (change accepted in July 2003): Form #184-Iron Ancillary Study Aliquot form must be completed and data entered. Data entry of this form will allow NERI to track the shipment and receipt of this specimen.

Batches of the liver biopsies from University of California, Irvine (25-50 / batch) will be shipped at ambient temperature by 2-4 day delivery to:

Richard W. Lambrecht, Ph. D
Univ. of CT Health Center
263 Farmington Ave
Farmington, CT 06031

Phone: 860-679-4886
Fax: 860-679-1931
Email: lambrect@uchc.edu

E-mail or Fax Dr. Lambrecht prior to sending a shipment so he will be aware that a shipment is due to arrive.

Liver biopsies done at University of Connecticut/University of Massachusetts will be stored in UMASS/UCONN HALT-C freezers (-80º C) until they are assayed by Dr. Lambrecht. After specimens have been analyzed, Form # 182 Hepatic Iron Concentration has to be data entered into the HALT-C Data Management System at University of Connecticut / University of Massachusetts.

C. DNA

The Central Repository will isolate DNA from transformed EBV cell lines for all patients enrolled into HALT-C who have consented to participate in genetic studies.

University of Connecticut / University of Massachusetts will obtain DNA (3-4 mcg/patient) from the Central Repository, which will send frozen samples to University of Connecticut / University of Massachusetts in batches of 50-100. These samples will be used for *HFE* mutational analyses. DNA should be shipped to arrive Monday – Thursday only.

Specimens should be shipped to:

Richard W. Lambrecht, Ph. D
Univ. of CT Health Center
263 Farmington Ave
Farmington, CT 06031

Phone: 860-679-4886
Fax: 860-679-1931
Email: lambrect@uchc.edu

Prior to shipping DNA, call, fax, or e-mail Dr. Lambrecht so he will be looking for the shipment to arrive. Data set with HFE Gene Testing results will be sent to NERI and imported into the HALT-C Data Management System.

VI. Quantitative Iron Determination Assay Protocol:

Quantitative Iron Determination in Tissue Biopsies by Absorption Spectrophotometry of Fe-PAR Complex

Principle:

Acid digestion of tissue to quantify iron spectrophotometrically using a chelator that forms a stable colored compound.

Theory:

The spectrophotometric determination of iron in aqueous solutions relies on the formation of stable iron complexes possessing a high absorptivity in the visible region of the spectrum. A number of sensitive iron ligands have been developed over the years: the majority have been compounds possessing the ferroin group, i.e. the group –N=C-C=N-, which acts as a bidentate ligand towards iron II. In this method we use 4-(2-Pyridylazo)resorcinol (PAR), which has a molar absorptivity of 56,000 at 500 nm.

This chemical method was used to determine total liver-iron concentration in needle biopsy specimens from patients with various iron-loading disorders and with chronic liver disease. Diethylenetriamine penta-acetic acid (DPTA) chelatable iron, which provides an indirect quantitative estimate of total body iron stores, was also determined in most of the cases. There was a high degree of correlation between liver-iron concentration and DPTA-chelatable iron. Determination of the iron concentration in needle-biopsy specimens is a simple and practicable method for estimating tissue iron stores. The findings in patients with idiopathic and secondary hemochromatosis, and in those with other disorders, were closely correlated with the estimates of total body storage iron based on a chelation test using DPTA. The method does not measure non-heme iron selectively, however, and may therefore be imprecise under conditions of iron depletion.

Needle biopsy liver tissue is weighed and transferred to a drying oven at 80^{0}C for four hours or overnight. It is then ashed with a mixture of concentrated sulfuric and nitric acids in a 30 mL Kjeldahl flask. The resulting solution is treated with phosphate buffer and ammonium hydroxide, and the iron is reduced by hydroxylamine. The ferrous iron is complexed to PAR and absorbance of the complex is read on a spectrophotometer at 500 nm.

Reagents:

6N Nitric Acid
0.1% PAR
Digestion Mixture 50% (v:v) sulfuric and nitric acid
6M Ammonium Hydroxide
10% Hydroxylamine
0.5M Potassium Phosphate monobasic
0.5M Potassium Phosphate dibasic
0.5M Potassium Phosphate buffer
Primary Stock $FeCl_3$ Standard (1000 ppm or 0.1% or 1 mg/mL solution)
Secondary Stock $FeCl_3$ Standard (100 ug/mL)

Other Equipment:

Heating Cup
Kjeldahl 30mL tubes
Powerstat variable autotransformer
LKB 4050 spectrophotometer
16x100mm glass culture tubes
Drying Oven

Procedure:

NOTE: For samples in paraffin, cut the sample out of the block and place in a small weighing dish. Place the dish on a slant in the drying oven overnight. The next day, place the tissue in a glass scintillation vial and add 2mL of Xylene. Agitate sample and Xylene on a shaker for 3 hours at room temperature. Using a plastic pipet, remove as much xylene as possible. Place back into the drying oven for 1 hour. Start procedure below at step 3.

1. Number and record the weight of a scintillation vial (column 1), add a 20-30 mg piece of tissue, weigh and record the weight (column 2).
2. Place the scintillation vial containing the tissue in the Blue M drying oven for at least 2 hours at 80^0C.
3. Remove vial and let cool.
4. Record the weight of the scintillation vial and dried specimen (column 5).
5. Label Kjeldahl tubes for each control and samples.
6. Place dried sample into a 30mL Kjeldahl tube.
7. Record the weight of the scintillation vial and remaining sample (column 6)
8. Add 150uL of digestion mixture to all the Kjeldahl tubes.
9. Place Kjeldahl tube in the heating cup in the hood, and secure the tube to a clamp on metal stand
10. Set the variac to 60 and turn on. Heat for 3 minutes. If some tissue is still undigested, continue heating. It may be necessary to continue digesting under a slow stream of air. Cool.
11. Label 16x100mm tubes for working standard solutions and for each control and sample to be run.
12. Prepare secondary standard and working standards as follows:

Prepare the secondary stock standard by pipetting 1.0 mL of 1000 ppm stock into a 10.0mL volumetric flask and diluting to 10 mL with Milli Q water.

Value of Standard (ug Fe/tube)	mL of Secondary Stock 100 ug/mL	mL of Milli Q Water
0	0	4
5	1	3
10	2	2
15	3	1
20	4	0

Note: Working standards are prepared fresh on the day of use.

13. Pipet 200 uL of each working standard into the 16x100mm tubes.
14. Add 150 uL of digestion mixture to these tubes.
15. Add 2000 uL 0.5M potassium phosphate buffer to all tubes and Kjeldahl tubes.
16. Add 1750 uL 6M ammonium hydroxide to all tubes and Kjeldahl tubes (in hood).
17. Add 100 uL 10% hydroxylamine to all tubes and Kjeldahl tubes.
18. Add 5300 uL Milli Q water to all tubes and Kjeldahl tubes.
19. Pour the contents of each Kjeldahl tube into a 16x100mm tube.
20. Add 500 uL 0.1% PAR into each tube and invert gently.
21. Let tubes sit overnight in hood.
22. Zero the spectrophotometer with water and read the absorbance of the blank standard versus water and record. Zero the instrument using the blank and read the absorbances of the remaining standards and controls.
23. Graph the standard curve A500 vs. ug Fe/tube and calculate linear regression equation.
24. Calculate iron concentration in tissue as ug Fe/mg dry wt and calculate hepatic iron index using the formula listed in the Calculations section.

Quality Control:

Chicken embryo liver is run in triplicate with each assay as a positive control. The range for the liver is 0.25-0.6 ug Fe/mg dry liver and the control must be within this range to accept the run and report results.

Or,

2 freeze-dried chicken embryo liver homogenates are run in duplicate with each assay as a positive control. The range for the livers is 400-800 ug Fe/g dry liver and 800-1600 ug Fe/g dry liver. The controls must be within this range to accept the run and report results.

Calculations:

Record the weights in grams of the vial and liver as follows:

1	2	3	4	5	6
vial wt.	vial + wet liver	wet liver wt.	vial wt. + dry liver	wt. of vial remaining	dry liver wt

Wet liver wt. (column 3) = column 2 – column 1
Dry liver wt. (column 6) = column 4 – column 5

Next graph the standard curve A500 vs. ug Fe/tube and calculate the linear regression equation. Using the linear regression equation solve for “x” which gives ug Fe per sample:

ug Fe/tube (10mL) = (y + b) / m where y = A500, b = y-intercept, and m = slope
ug Fe/g dry wt = ug Fe / mg dry liver (column 6) x 1000 mg/g
umoles Fe/g dry wt = ug Fe/g dry wt / 55.85 ug Fe / umoles Fe
Hepatic Iron Index = umoles Fe / g dry wt / age of patient in years

Reference Ranges:
300-1200 ug Fe / g dry wt or 5.37 – 21.48 umoles Fe/ g dry wt.
Hepatic Iron Index: <1.5 (males) or <1.0 (females) umoles Fe/ g dry wt per year of age.

References:

1. Barry M, Sherlock S. Measurement of Liver-iron Concentration in Needle-biopsy Specimens. Lancet 1971; 1:100-103.
2. Vinogradov SF. Spectrophotometric Determination of Iron in Heme Proteins. Meth Enzymol 1986; 123:320-323.
3. Nonova D, Evtimova GJ. Complexing of Iron (II) and Iron (III) by 4-(2-pyridylazo)resourcinol. Inorg Nucl Chem 1973; 35:3581-3586.
4. Yotsuyanage T, Yamashita R, Aomura K. Highly Selective and Sensitive Spectrophotometric Determination of Iron (II) and Cobalt (III) with 4-(2-pyridylazo)resourcinol (PAR). Anal Chem 1972; 44:1091.
5. Ultrospec Instruction Manual, LKB, 1982.

VII. HFE Gene Analysis Assay Protocol:

HFE Gene Analysis for Detection of C282Y and H63D Mutations

Principle:

To detect HFE gene mutations in DNA isolated from cultured cells and non-coagulated whole blood by using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods based on those of Feder et al. [Nat Genet 1996 Aug;13(4):399-408]

Theory:

Two mutations in the HFE gene have been discovered which are believed to be responsible for the iron overload syndrome seen in many patients with genetic hemochromatosis. These mutations, designated C282Y and H63D, correspond to a tyrosine for cysteine substitution, and aspartate for histidine substitution, respectively. The areas in the gene containing these mutations can be amplified by PCR using primer sequences reported by Feder et al and Jeffrey et al. The amplified portions are then cut with restriction enzymes and run on an agarose gel to yield a pattern of bands characteristic of homozygous normal, heterozygous, or homozygous abnormal genotypes.

Chemicals and Supplies:

DNA Isolation Procedure:

- 200 uL whole blood containing EDTA as an anticoagulant or 200 uL cultured cells
- Qiagen Blood Kit (for isolation of DNA from whole blood or cultured cells)
- 100% Ethanol
- 1.5 mL microfuge tubes
- filtered pipet tips

PCR Procedure:

- 25 uL DNA isolated from Qiagen Kit (for samples and for controls)
- 25 pmol/uL forward and reverse primers:
  - C282Y forward sequence 5' TGG CAA GGG TAA ACA GAT CC 3'
  - C282Y reverse sequence 5' TAC CTC CTC AGG CAC TCC TC 3'
  - H63D forward sequence 5' ACA TGG TTA AGG CCT GTT GC 3'
  - H63D reverse sequence 5' GCC ACA TCT GGC TTG AAA TT 3'
- High quality water (>17 mΩ)
- Pharmacia Biotech Ready-to-Go PCR Beads (which contain standard concentrations of Taq Polymerase, dNTPs, MgCl2 upon rehydration of the bead)
- Mineral oil
- Perkin Elmer Thermalcycler

RFLP Procedure:

- 15 uL of PCR products previously amplified (from samples and controls)
- Master Mix containing specific restriction enzyme buffer, BSA, and Milli Q water
- Restriction Enzyme SnaB1 (for C282Y mutation) and Bcl 1 (for H63D mutation)
- Loading Dye (1X)
- Ethidium Bromide
- BioRad High Grade Agarose (1.5g per 50 mL gel = 3% agarose gel)
- 1X TAE buffer (Tris and EDTA containing buffer)
- 50 mL gel apparatus with two 8-well combs per gel
- Power source to generate 100 volts to the gel apparatus
- Polaroid Camera and film, UV light filter, orange-colored filter

Procedure:

DNA Isolation

1. Using the Qiagen Blood Kit for DNA isolation, add 20 uL Protease to each sample in a labeled 1.5 mL microfuge tube.
2. Add 200 uL Lysis Buffer to a tube then pulse vortex for 20-30 seconds; repeat for each tube.
3. Gently spin tubes to remove liquid from tube lids.
4. Incubate at 55^{0}C for 10 minutes.
5. Gently spin tubes to remove condensation from tube lids.
6. Add 200 uL 100% Ethanol; vortex 5 seconds.
7. Gently spin tubes.
8. Transfer the volume from the tube into a labeled spin column containing a flow through capture vessel.
9. Centrifuge the spin column at approx 10,000xg for 1 minute.
10. Remove the flow through vessel and replace with a clean one.
11. Add 500 uL Wash Buffer 1; Centrifuge at 10,000xg for 1 minute.
12. Remove flow through vessel and replace with clean one.
13. Add 500 uL Wash Buffer 2; Centrifuge at 10,000xg for 3 minutes.
14. Remove flow through vessel and replace with a labeled clean 1.5 mL microfuge tube.
15. Add 200 uL Elution Buffer; let stand for 1 minute at room temperature.
16. Centrifuge at 10,000xg for 30 seconds .
17. Eluate contains the DNA; discard spin column.
18. DNA can then be frozen at -20^{0}C and thawed later, or used immediately for PCR.

PCR

NOTE: Only one region of the gene can be amplified per Ready-To-Go PCR Bead, therefore, you will need to run 2 beads for each sample or control; one with the primers for the C282Y mutation and one with the primers for the H63D mutation. In total you will be using 46 uL DNA per sample or control.

1. Label two tubes containing a Ready-to-Go PCR Bead for each sample or control.
2. Add 23 uL DNA isolated from Qiagen Kit into each tube.
3. For the No DNA control tube, add 23 uL Milli Q water instead of DNA.
4. Add 1 uL of 25 pmol/uL C282Y forward primer to one set of tubes.
5. Add 1 uL of 25 pmol/uL C282Y reverse primer to same set of tubes.
6. Add 1 uL of 25 pmol/uL H63D forward primer to other set of tubes.
7. Add 1 uL of 25 pmol/uL H63D reverse primer to same set of tubes.
8. Mix contents of tube by flicking tube 3 times, then gently spin down volume.
9. Gently add 50 uL mineral oil to the top of the mixture.
10. Run samples through a pre-set program on the Perkin Elmer ThermalCycler as follows:
    - 94^0C for 1 minute
    - 94^0C for 30 seconds }
    - 63^0C for 30 seconds } 35 cycles
    - 72^0C for 1 minute }
    - 72^0C for 10 minutes
    - 4^0C soak

RFLP:

NOTE: Only one set of PCR products can be analyzed per tube, therefore, there will be 2 tubes per sample or control for each RFLP genotype determination.

1. Prepare the two separate Master Mix + Restriction Enzyme solutions. Multiply the number of tubes per mutational analysis by 9 to get the total volume in uLs of Master Mix needed (per mutation). Add this amount into a 500 uL snap-top tube.

   The *SnaB 1* package insert claims to digest 10 samples per unit of enzyme, and the enzyme concentration in the tube is usually 10 u/uL. The digests are more complete if 3-4x the amount of enzyme is used, therefore divide the number of samples and controls by 10, and multiply that number by 3 or 4 to determine how many uLs of enzyme to add to the C282Y Master Mix. Add this amount into the C282Y Master Mix previously aliquotted, then keep tube on ice.

   The *Bcl 1* package insert claims to digest 10 samples per unit enzyme, and the enzyme concentration in the tube is typically 10 u/uL. Digestion with this enzyme is very good, therefore, after dividing the number of samples and controls by 10, multiply that number by 2 or 3 to determine how many uLs of enzyme to add to the H63D Master Mix. Add this amount into the H63D Master Mix previously aliquotted, then keep tube on ice.
2. Label a set of 500 uL tubes (one per sample per mutation), and remove 15 uL of PCR product from each sample and control tube, and add to a 500 uL tube. Be careful to avoid drawing up the mineral oil. With a clean Kim-wipe, quickly swipe the end of the pipet tip after removal from the mineral oil phase in the PCR tube.
3. Add 9 uL of Master Mix + Enzyme into respective tubes, mix by flicking tube 3 times, then gently spin down the volume.
4. Incubate tubes for the C282Y analysis at 37^0C for 2 hours, and the tubes for the H63D analysis at 50^0C for 2 hours.

5. Prepare agarose gels 1/2 hour before incubation is complete:
   1-3% agarose gel with two 8-well combs holds 14 controls and samples, and the 2 remaining wells (one at the beginning of each row) is for the Phi X lane marker. Divide the total number of samples and controls by 14 to determine the number of gels to be prepared.

   Per gel, weigh 1.5 g of BioRad High Grade Agarose. Add agarose to 50 mLs of 1X TAE Buffer in an Erlenmeyer flask. Melt agarose in microwave on high for 1 minute.
   Add 0.8 uL Ethidium Bromide to the melted gel solution, and pour into gel apparatus. Add combs and let solidify for 1/2 hour.
6. Add 3 uL 1X loading dye to each digest tube, flick 3 times, spin down volume.
7. Prepare Phi X marker tubes by adding 1.5 uL Phi X marker to 23 uL Milli Q water, and then add 3 uL 1X loading dye. Flick 3 times, spin down volume.
8. Add 1X TAE to the gel apparatus to completely cover the gel and fill gutters on each side. Load samples in each well, run gel at 100 volts for 30 minutes.
9. Remove gel and visualize bands using a UV transilluminator, then take picture of gel using a Polaroid camera and film.
10. Determine genotype by looking at pattern of bands:
    C282Y RFLP Band Patterns (using *SnaB1*):
    Homozygous normal ~ one band at 390 bp
    Heterozygous ~ three bands at 390, 277, 113 bp
    Homozygous abnormal ~ two bands at 277 and 113 bp

    H63D RFLP Band Patterns (using *Bcl1*):
    Homozygous normal ~ two bands at 138 and 70 bp
    Heterozygous ~ three bands at 138, 70, and 208 bp
    Homozygous abnormal ~ one band at 208 bp

References:

1. Feder JN et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nature Genetics 1996 Aug;13(4):399-408.
2. Jeffrey GP et al. Polymorphism in intron 4 of HFE may cause overestimation of C282Y homozygote prevalence in haemochromatosis. Nature Genetics 1999 Aug;22(4):325-326.
3. Qiagen QIAamp Blood Mini Kit Handbook, January 1999.