APPENDIX A: HALT-C Ancillary Study PROPOSAL (Revision #3)

Part I (1 page)
Proposal Title: Analysis of keratin genetic variants and Mallory-Denk bodies in patients with chronic hepatitis C

Proposal PI: M. Bishr Omary PhD, MD

Co-Investigators: Herbert L. Bonkovsky MD (HALT-C investigator), Robert Fontana MD (HALT-C investigator), Kristin Snow PhD (NERI investigator), Elizabeth C. Wright PhD (NIH representative and senior statistician), Mina O. Rakoski MD (Gastroenterolgy fellow, University of Michigan), Natasha Snider (postdoctoral fellow, University of Michigan)

HALT-C PI: Herbert L. Bonkovsky MD

Funding Agency and Review Body: Institutional funds of the University of Michigan

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

_____	_____
Proposal Principal Investigator	Date
_____	_____
HALT-C Principal Investigator	Date

Protocol Part II

1. Aims/hypotheses: Recent literature has begun to uncover the role of keratin proteins in protecting cells from apoptosis and in the formation of Mallory-Denk bodies (MDBs, formerly known as Mallory bodies) that are seen in association with several liver disorders, particularly steatohepatitis but also hepatitis C and hepatocellular carcinoma. In addition, patients with keratin polymorphisms appear to be predisposed to liver disease progression and increased fibrosis. The first aim of this protocol investigates the association of keratin genetic variants with the severity of chronic hepatitis C and response to therapy. One of our two central hypotheses is that keratin polypeptide 8 and 18 (K8/K18) specific variants predispose to hepatitis C liver disease progression and may modulate response to therapy.

The second aim of this proposal evaluates the association between risk factors such as age (which may related to oxidative stress exposure) and gender and the formation of MDBs in patients with chronic hepatitis C. Several modifier genes such as K8, p62, TG2 are known to be essential for MDB formation. Our second central hypothesis is that formation of MDBs are related to specific patient

characteristics such as gender and age, and may be more prominent in males and patients with older age.

<u>Aim 1:</u> Association of keratin genetic variants with the severity of chronic hepatitis C and response to therapy

Aim 1.1: Determine the prevalence of K8 and K18 exonic and intron-exon boundary variants in the entire Halt-C patient cohort. ***Hypothesis 1.1****:* K8 and K18 variants are overrepresented in patients with more rapidly progressive chronic HCV infection and may also be overrepresented in patients who are less responsive to therapy with pegylated interferon and ribavirin. Progressive HCV infection implies patients who have experienced one or more outcomes, especially those who have died of liver disease, developed hepatocellular carcinoma, or have required liver transplants. Less rapidly progressive chronic HCV infection reflects those who have not experienced outcomes, death, HCC, or liver transplant. With regard to studying the potential role of keratin mutation and response to therapy, we will compare the distribution of keratin variants in patients who achieved an SVR in the lead-in phase versus those who did not.

Aim 1.2: Determine if unique K8 or K18 variants segregate with specific ethnic backgrounds. ***Hypothesis 1.2:*** Specific K8 and K18 variants are overrepresented in African American patients and may correlate chronic HCV infection disease severity or response to therapy.

Taken together, these two aims will provide a detailed and comprehensive analysis regarding the possible role of keratin polymorphisms in the progression of chronic hepatitis C and response to antiviral therapy**.** We predict that our findings will enhance our limited understanding of the genetic modifiers that impact on HCV infection and response to therapy. The importance of keratin variants in liver disease progression is supported by two animal transgenic mouse models that express the keratin polypeptide 8 (K8) variants Gly61Cys (1) or Arg340His (2) which are significantly more predisposed to liver injury as compared with the wild-type counterparts.

Aim 1. Background and rationale: There is accumulating evidence that modifier gene variants can play an important role in modulating the severity and response to therapy in patients with hepatitis C virus (HCV)-related liver disease. One example of such modifier gene is *HFE* and in particular the H63D *HFE* variant which appears to afford a significant benefit in the sustained virologic response [eg, 20% in those with *HFE* mutation compared with 14% in those without mutation, p=0.009 (3)]. Other attractive candidate modifier gene products are keratin polypeptides 8 and 18 (K8/K18) which are known to protect the liver from a variety of toxic insults such as hepatotoxins (eg, acetaminophen or agents that induce apoptosis) [reviewed in ref. 4] or thioacetamide in the context of a mouse fibrosis model (5). These keratins are involved in a variety of cellular functions including regulating susceptibility to undergo apoptosis, protein targeting to subcellular compartments (eg, apical versus basolateral domains) and the regulation of cell signaling [reviewed in ref.4). Carriers of heterozygous K8/K18 variants have an odds ratio of 3.7 (95% CI=2-6.8) of having end-stage liver disease (assessed using liver explants) which in relative terms is higher than the risk of acute myocardial infarction in smokers of 10-19 cigarettes/day [as compared with nonsmokers, odds ratio of 2.6 (95% CI=2.4-2.9)]. In addition, certain keratin variants segregate exclusively with specific ethnic backgrounds as exemplified by the identification of the K8 G434S variant in 10 of 41 (24.4%) African American patients with acute liver failure but in only 25 of 245 (10.2%) blood bank African American controls ($p<0.02$; OR 2.8, 95%CI 1.1-6.8) and in none of >700 tested individual of other ethnic backgrounds. Notably, the human association studies are supported by extensive studies using genetic keratin-mutant animal models (4).

With regard to patients with HCV infection, the only available keratin-related study (carried out in a German cohort of 329 patients with chronic HCV infection) showed that specific keratin variants are over-represented in patients with advanced HCV infection (6). This finding makes the Hepatitis C Anti-

viral Long-term Treatment to prevent Cirrhosis (Halt-C) trial patient cohort ideal to further test the importance of keratin variants in HCV-related liver disease progression and response to therapy. In addition, the availability of patients with diverse ethnic backgrounds in the Halt-C study cohort (eg 15% African Americans from the total cohort of >1000 enrollees) will provide the means to assess the potential significance of unique keratin variants that are found exclusively in African Americans.

Aim 1. Relations to aims of HALT-C study: We submit this proposal at the recommendation of the Genomics-Genetics Committee. In a recent meeting of this Committee, during which the findings to date of studies of genetic associations with severity, progression, and response to therapy of chronic hepatitis C were discussed, Dr. Bonkovsky, the Chair of the Committee, was charged with trying to recruit the interest and effort of Dr. Omary to investigate the possible roles of genetic variations in keratins 8 and 18. This recommendation is based upon the body of work of Dr. Omary and his team during the past several years, establishing that such genetic variations are important in chronic liver diseases [reviewed in Ref. 4, 7].

Aim 1. Study design, experimental groups: In order to minimize bias, and in order to take advantage of our high throughput methods of analysis, we anticipate studying DNA from the entire cohort of Halt-C patients who have given consent for genetic tests to be performed. Therefore, we anticipate ~1000 subjects from whom genomic DNA is available. Based on the extensive studies we have carried out to date, we will need 1-1.5 μg of genomic DNA. Initially we request 1 μg of DNA from the entire cohort of patients who have consented to undergo genetic testing although our primarly group of analysis will be the 951 randomized patients who have outcome data. A total of 15 exons will be analyzed, which represents the entire coding regions of K8 and K18. Any unused DNA will be returned to the HALT-C DNA repository.

Aim 1. Methods, data usage: The methods will be those already in place and routine use in Dr. Omary's laboratory. They involve amplification of all 15 exons and the Intron-exon boundaries of the *KRT8* (K8) and *KRT18* (K8) genes. Amplified amplicons will be analyzed using denaturing high-performance liquid chromatography (DHPLC) which is a high throughput method that we have been using for the last 5 years. This method allows the analysis of 12 samples per hour and remains state-of-the-art and we have used it reliably and have defined experimental conditions that allow mutation detection (8) that others have missed (9). Samples that elute off the DHPLC column will a shifted pattern suggest the presence of a mutation and will be sequenced. Depending upon what is found, genetic variations that appear likely from in silico analyses to be of functional importance may be expressed and phenotypes assessed in suitable in vitro expression systems.

Aim 1. Anticipated results: We expect to find genetic variations in K8 and/or K18 that are associated with severity, progression, and potentially response to therapy of advanced chronic hepatitis C.

Aim 1. Statistical support: This may be provided either by Drs. Snow or Wright and their teams or by Dr. Lazzeroni, a biostatistician with whom Dr. Omary has worked for several years at Stanford (eg, ref 2,6). Dr. Lazzeroni has offered her support in regards to the statistical analysis for this study (see attached letter).

Aim 1. HALT-C samples to be used in the study: Initially 1.0 μg of genomic DNA will be requested. If additional material should be needed an additional 0.5 μg will be sought (total of no more than 1.5 μg).

Aim 1. Financial issues: We will pay for costs, if any, for samples to be retrieved from the repository and shipped to Dr. Omary's laboratory at the University of Michigan. If additional costs for data analyses are felt to be required by NERI, we will instead execute a data sharing agreement, so that analyses can be performed by our team of biostatisticians.

Aim 1. References:

1. Ku N-O and Omary MB. (2006). A disease and phosphorylation related non-mechanical function for keratin 8. *J Cell Biol* 174:115-25.
2. Strnad P, Zhou Q, Hanada S, Lazzeroni LC, Zhong BH, So P, Davern TJ and Lee WM for the Acute Liver Failure Study Group (ALFSG), Omary MB. Ethnic-specific keratin variants predispose to the development of acute liver failure. *Submitted*.
3. Bonkovsky HL et al (2006). Roles of iron and HFE mutations on severity and responses to therapy during retreatment of advanced chronic hepatitis C. *Gastroenterology* 131:1440-1451.
4. Ku N-O, Strnad P, Zhong B, Tao GZ and Omary MB (2007). Keratins let liver live: mutations predispose to liver disease and crosslinking generates Mallory-Denk bodies. *Hepatology* 46:1639-49.
5. Strnad P, Tao G-Z, Zhou Q, Harada M, Toivola DM, Brunt EM and Omary MB. (2008). Keratin mutation predisposes to mouse liver fibrosis and unmasks differential effects of the CCl_4 and thioacetamide models. *Gastroenterology* 134:1169-1179.
6. Strnad P, Lienau TC, Tao G-Z, Lazzeroni LC, Stickel F, Schuppan D and Omary MB (2006). Keratin variants associate with progression of fibrosis during chronic hepatitis C infection. *Hepatology* 43:1354-63.
7. Omary MB, Ku NO, Strnad P, Zhong B, Tao GZ (2009). Towards unraveling the complexity of 'simple' epithelial keratins in health and disease. *J Clin Invest*, in press.
8. Strnad P, Lienau TC, Tao G-Z, Ku N-O, Magin TM and Omary MB (2006). Denaturing temperature selection may underestimate keratin mutation detection by DHPLC. *Human Mutat* 27:444-452.
9. Hesse M, Berg T, Wiedenmann B, Spengler U, Woitas RP, Magin TM. A frequent keratin 8 p.L227L polymorphism, but no point mutations in keratin 8 and 18 genes, in patients with various liver disorders. *J Med Genet* 2004;41:e42

<u>Aim 2:</u> Analysis of the risk factors associated with the presence of Mallory-Denk Bodies

Aim 2.1: Determine the association between risk factors such as gender and age and the formation of Mallory-Denk bodies (MDBs)
Hypothesis 2.1: The formation of MDBs is related to specific patient characteristics such as gender and age, and may be more prominent in males and patients with older age.

The comprehensive HALT-C database allows analysis of MDB formation in different patient populations, while accounting for confounders such as BMI, alcohol, diabetes, steatosis and other histological scores. If this hypothesis is proven correct, it has potential direct clinical relevance given the increased rate of progression of alcohol-related liver injury in women. Our hypothesis is supported by findings in animal models that are summarized in an abstract that has been submitted to the 2009 DDW which has been selected as "poster of distinction" (see bottom of reference section).

Aim2. Background and rationale: Mallory-Denk bodies (MDB) are hepatocyte cytoplasmic inclusions found in several chronic liver diseases, including alcoholic and nonalcoholic steatohepatitis, hepatitis C and hepatocellular carcinoma. Keratin polypeptides 8 and 18 (K8/K18) comprise the primary constituents of Mallory-Denk bodies (MDBs). Increased K8>K18 ratio as a result of cell injury as well as keratin crosslinking by transglutaminase-2 play essential roles in MDB formation (1). Recent studies in animals indicate that the genetic background and gender can play a critical role in experimental formation of MDBs (2,3). MDB formation is reversible after discontinuation of alcohol or the inciting drug, but can rapidly reform upon re-exposure to toxic insult. Refining our understanding of the precise mechanism and genetics of MDB formation should help elucidate whether MDBs serve a protective cell response, and whether they offer prognostic or diagnostic value. The precise risk factors that lead to MDB formation as well as its role in liver disease severity or progression is not fully understood. The current understanding of MDB formation is summarized in two recent reviews: (a) Zatloukal K, Denk H, Stumptner C, Strnad P, Harada M, Toivola DM, Cadrin M and Omary MB (2007). From Mallory to Mallory-Denk bodies: What, how and why? *Exp Cell Res* 313:2033-2049. (b) Ku N-O,

Strnad P, Zhong B, Tao GZ and Omary MB (2007). Keratins let liver live: mutations predispose to liver disease and crosslinking generates Mallory-Denk bodies. *Hepatology* 46:1639-1649.

Our group has carried out several studies pertaining to understanding the molecular factors related to MDB formation (this work is currently supported by an active R01: DK52951). These studies have led to several publications by our group and in collaboration with others (please see reference section).

Aim2. Relations to aims of HALT-C study: The HALT-C cohort provides an ideal group to study gender, age and other potential confounder variables related to MDB formation.

Aim2. Study design, experimental groups: We will utilize the demographic, clinical, serologic and histologic data from all patients in the HALT-C trial for a critical analysis of predictors associated with development of MDBs.

Aim2. Methods, data usage: The large collection of data will be analyzed using univariate, multivariate and logisitic regression analysis.

Aim2. Anticipated results: If the animal data apply to humans, we anticipate at the very least to confirm that males are more prone to MDB formation. In addition, we hope to capitalize on the power of the available data to potentially develop hypothesis regarding other potential associations that we can then test in animals and work towards understanding their mechanism.

Aim2. Statistical support: This will be provided either by Dr. Lazzeroni, a biostatistician who has collaborated with us (as Discussed for Aim1; please see attached letter). There are also robust statistical analysis capabilities here at the University of Michigan that we may use as the need arises.

Aim2. HALT-C samples to be used in the study: No specimens from the HALT-C trial will be utilized at this stage. Should our data analysis require this, we will request such use.

Aim2. Financial issues: None anticipated. All costs will be covered by Dr. Omary existing funds.

Aim2. References:

1. Zatloukal K, French SW, Stumptner C, Strnad P, Harada M, Toivola DM, et al. From Mallory to Mallory-Denk bodies: What, how and why? *Exp Cell Res* 2007; 313: 2033-2049.

2. Hanada S, Strnad P, Brunt EM, Omary MB. Male predisposition to mouse Mallory-Denk body formation is reversed by esterdiol. Abstract DDW 2009.

3. Hanada S, Strnad P, Brunt EM, Omary MB. The genetic background modulates susceptibility to mouse liver Mallory-Denk body formation and liver injury. *Hepatology* 2008;48:943-952.

Publications by our group, or with collaboration, pertaining to understanding the dynamics of MDB formation:

1. Stemptner C, Omary MB, Fickert P, Denk H, and Zatloukal K (2000). Hepatocyte cytokeratins are hyperphosphorylated at multiple sites in human alcoholic hepatitis and in a Mallory body mouse model. Am J Pathol 156:77-90.

2. Harada M, Kumemura H, Omary MB, Kawaguchi T, Maeyama N, Hanada S, Taniguchi E, Koga H, Suganuma T, Ueno T, and Sata M (2003). Proteasome inhibition induces inclusion bodies associated with intermediate filaments and fragmentation of the Golgi apparatus. Exp Cell Res 288:60-69.

3. Hanada S, Harada M, Kumemura H, Omary MB, Kawaguchi T, Taniguchi E, Koga H, Maeyama M, Yoshida T, Baba S, Ueno T, and Sata M (2005). Keratin-containing inclusions affect cell morphology and distribution of cytosolic cellular components. Exp Cell Res 304:471-482.

4. Nakamichi I, Toivola DM, Strnad P, Michie SA, Oshima RG, Baribault H and Omary MB (2005). Keratin 8 overexpression accelerates mouse Mallory body formation. J Cell Biol 171:931-937.

5. Strnad P, Siegel M, Toivola DM, Choi K, Kosek JC, Khosla C and Omary MB (2006). Pharmacologic transglutaminase inhibition attenuates drug-primed liver hypertrophy but not Mallory body formation. FEBS Lett 580:2351-2357.

6. Harada M, Strnad P, Resurreccion EZ, Ku NO and Omary MB. (2007). Keratin 18 overexpression but not phosphorylation or filament organization blocks mouse Mallory body formation. Hepatology 45:88-96.

7. Strnad P, Harada M, Siegel M, Terkeltaub RA, Graham RM, Khosla C, and Omary MB (2007). Transglutaminase 2 regulates Mallory body inclusion formation and injury-associated liver enlargement. Gastroenterology 132:1515-1526.

8. Hanada S, Harada M, Kumemura H, Omary MB, Koga K, Kawaguchi T, Taniguchi E, Yoshida T, Hisamoto T, Yanagimoto C, Maeyama M, Ueno T, and Sata M (2007). Oxidative stress induces the endoplastic reticulum stress and facilitates inclusion body formation in cultured cells. J Hepatol 47:93-102.

9. Harada M, Strnad P, Toivola DM, Omary MB. (2008). Autophagy modulates inclusion formation and apoptosis in cell culture: Inclusions associate with organelle reorganization. Exp Cell Res 314:1753-1764.

10. Harada M, Hanada S, Toivola DM, Ghori N, Yoshimori T and Omary MB. (2008). Autophagy activation by rapamycin eliminates mouse Mallory-Denk bodies and blocks their proteasome inhibitor-mediated formation. Hepatology 47:2026-2035.

11. Strnad P, Tao G-Z, So P, Lau K, Schilling J, Wei Y, Liao J, Omary MB. (2008). 'Toxic memory' via chaperone modification is a potential mechanism for rapid Mallory-Denk body re-induction. Hepatology 48:931-942.

12. Hanada S, Strnad P, Brunt EM, Omary MB. (2008). The genetic background modulates susceptibility to mouse liver Mallory-Denk body formation and liver injury. Hepatology 48:943-952.

Below is an abstract submitted by our groups to the 2009 DDW:

Abstract Title: Male predisposition to mouse Mallory-Denk body formation is reversed by esterdiol
Abstract Authors: Shinichiro Hanada, Elizabeth M Brunt[1] and M Bishr Omary

Department of Molecular & Integrative Physiology, University of Michigan, 7744 Medical Science Building II, 1301 E. Catherine, Ann Arbor, MI 48109-0622; and [1]Department of Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid Ave, Campus Box 8118, St. Louis, MO 63110

Background: Mallory-Denk bodies (MDBs) (formerly known as Mallory bodies) are hepatocyte cytoplasmic inclusions found in several liver diseases particularly alcohol-related liver injury. MDBs consist primarily of keratins 8 and 18 (K8/18), which are the cytoskeletal intermediate filament proteins of hepatocytes. Several genetic mouse studies demonstrated that MDB formation requires a K8>K18 overexpression state and transamidation via transglutaminase-2 in response to liver injury. The genetic background plays an important role in MDB formation, but the effect of gender on inclusion formation is unknown. Also, it is unknown whether MDBs protect or propagate liver injury. Aims: Given that significant difference in predisposition to liver injury among males and females, we hypothesized that gender differences also contribute to the extent of MDB formation. Methods: We used an established mouse MDB model that we previously characterized (J Cell Biol 2005), that involves feeding (for 6 weeks) transgenic mice that over-express human K8 (or that over-express mouse K8) 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). MDB formation was assessed using histology and immunofluorescence staining with antibodies to K8/K18, ubiquitin, and the ubiquitin-binding protein p62; and biochemically by demonstrating keratin-containing crosslinks generated by

transglutaminase-2. Estradiol (10 μg/kg, 3 days/week; or carrier) was also administered to male and female mice to test their effect on MDB formation. Results: DDC feeding induces MDB formation and keratin filament disruption in both males and females, but there is a dramatic increase in MDB formation in males as determined using immunofluorescence (125.2 ± 19 in males versus 68.8 ± 19.4 in females; $p<0.01$) and histochemical (22.7 ± 13.2 in males versus 13.3 ± 11.9; $p<0.05$) staining. Histological analysis also demonstrated increased ductular reaction in the female mice. Biochemical characterization showed that MDB formation paralleled the generation of high molecular weight ubiquitinated keratin-containing complexes, but there was no difference in transglutaminase-2 induction between genders. Estradiol treatment of male mice decreased the number of generated MDBs as compared with vehicle-treated animals (18.9 ± 6.8 in males given vehicle versus 12.5 ± 4.7 in males given estradiol; $p<0.01$). Conclusions: Our findings show that male mice are markedly more susceptible to MDB formation, but this susceptibility decreases significantly upon estrogen supplementation. The increased MDB formation in males suggests a cell protective role for these inclusions and raises that possibility of similar findings in humans.

Protocol Part III: Sample Requirements.

Aim 1:

Visit	Liver # patients, mm*	Blood # patients, ml	DNA # patients, ug	Liver Biopsy Slides # patients, slides/patient	Other (describe) # pts, amount
Screen 1					
Screen 2			~1050—all who consented to undergo genetic testing and for whom there is DNA available		
Baseline			A total of 1 but no more than 1.5 μg of genomic DNA, which has been collected		
Lead in Week 4					
Week 8					
Week 12					
W16					
Week 20					
Week 24					
Randomized Month 9					
Month 12					
Month 15					
Month 18					
Month 21					
Month 24					
Month 27					
Month 30					

Month 33					
Month 36					
Month 39					
Month 42					
Month 45					
Month 48					
Post-treatment					
Responders W30					
W36					
W42					
W48					
W60					
W72					

* Assume 1 mm tissue weighs about 0.75 mg (= 0.5 mm^2 X Π X density of tissue)

Data needed (please specify): (requested after discussion with Kristin Snow)
Aims 1 and 2:

- Demographics, duration of HCV infection, alcohol and smoking history, BMI, HCV genotype, diabetes history
- Baseline information: histology (date of biopsy, inflammation, fibrosis, iron and steatosis score, Mallory-Denk bodies, pericellular fibrosis, length of biopsy and fragmentation), baseline labs (date of labs, CBC, liver panel, Cr, glucose, insulin, INR, AFP, HCV RNA), baseline ultrasound (date of ultrasound or other imaging, spleen size), baseline EGD (date of EGD, presence or absence of esophageal varices / portal gastropathy)
- Month 24 information: histology (date of biopsy, inflammation, fibrosis, iron and steatosis score, Mallory-Denk bodies, pericellular fibrosis, length of biopsy and fragmentation), labs (date of labs, CBC, liver panel, Cr, glucose, insulin, INR, AFP, HCV RNA), ultrasound (date of ultrasound or other imaging, spleen size)
- Month 48 information: histology (date of biopsy, inflammation, fibrosis, iron and steatosis score, Mallory-Denk bodies, pericellular fibrosis, length of biopsy and fragmentation), labs (date of labs, CBC, liver panel, Cr, INR, AFP, HCV RNA), ultrasound (date of ultrasound or other imaging, spleen size), EGD (date of EGD, presence or absence of esophageal varices / portal gastropathy)
- Information at last follow-up: labs (date of labs, CBC, liver panel, Cr, INR, AFP, HCV RNA), ultrasound (date of ultrasound or other imaging, spleen size)
- Treatment assignment: peginterferon vs. control, for peginterferon group – date of last dose
- Occurrence of clinical outcomes: date when each outcome is reached, for HCC – tumor stage

We also request access to the following data from the HALT-C Main Trial:

- Hepatic Steatosis (glycosylated hemoglobin, fasting insulin)
- Iron-related testing (histopathology, serum iron, HFE genetic testing)
- Pre-Study Serial Histology (past biopsy data)