APPENDIX A: HALT-C Ancillary Study PROPOSAL (Version 8/19/08)

Part I (1 page)

Proposal Name: ***Genome-wide Association Study of Depression and Suicidal Ideation Emerging during Interferon Treatment***

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HALT-C PI: Marc Ghany

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

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.

_____	8/19/08
Proposal Principal Investigator	Date
_____	_____
HALT-C Principal Investigator	Date

Protocol Part II (4 page limit, single space)

1. Aims/hypotheses
2. Background/rationale
3. Relations to aims of HALT-C study
4. Study design, experimental groups
5. Methods, data usage
6. Anticipated results
7. Statistical support
8. HALT-C samples to be used in the study (complete Part III: Sample Requirements)
9. Financial issues (e.g., cost for data analysis and obtaining samples from Repository)
10. References

Protocol Part III: Sample Requirements. (link to web site with actual sample availability)

Visit	Liver # patients, mm*	Blood # patients, ml	DNA # patients, ug	Liver Biopsy Slides # patients, slides/patient	Other (describe) # pts, amount
Screen 1					
Screen 2					
Baseline			1119, 2ug		
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): please see section VI

Comments (if any): none

SECTION I. SPECIFIC AIMS/HYPOTHESES

This study will investigate the relationship between genetic markers and the emergence of depressive symptoms and suicidal thoughts/behavior during alpha-interferon treatment. Our *long-term goal* is to understand the underlying pathophysiology of interferon treatment emergent depression, suicidal ideation or behavior and their genetic basis, in order that improved and more personalized therapeutic interventions can be developed. The *objective* of this application is to search the entire human genome for genetic variation that is associated with symptoms of depression and suicidal ideation or behavior that develop during interferon/ribavirin treatment of patients with hepatitis C viral (HCV) infection. Our *central hypothesis* is that genetic markers associate with interferon/ribavirin emergent depressive symptoms and suicidal ideation or behavior. The *rationale* for this study is derived from two studies which we led at the NIMH Intramural Research Program and the established finding that depressive symptoms and suicidal thoughts/ behavior can be provoked by interferon treatment. We have described: 1) association between genetic variation in GRIK2 and GRIA3 and citalopram treatment-emergent suicidal ideation (TESI) in the STAR*D sample (1); and 2) association between genetic variation in PAPLN and IL28RA and TESI in the same sample (2). IL28RA encodes an interleukin receptor for interferon lambda which is in the same cytokine family as alpha-interferon. These findings suggest that these genes could be influencing suicidal ideation emerging during treatment with drugs that have systemic effects on serotonin and interleukin signaling. This raises the question of whether a functional relationship exists between the described genes and the onset of depressive symptoms and ideas of suicide. Our **Specific Aims** are:

#1 Test if genetic markers associate with interferon/ribavirin treatment emergent or worsening symptoms of depression. We will search the entire genome for genetic markers with predictive power to detect emerging or worsening symptoms of depression.

#2 Confirm and characterize the association between genetic variation in GRIK2, GRIA3, PAPLN and IL28RA and treatment-emergent suicidal ideation in patients treated with interferon/ribavirin. Our Preliminary Studies have shown that genetic variation in GRIK2, GRIA3, PAPLN and IL28RA is associated with treatment-emergent suicidal ideation. We aim to test this association in an independent cohort treated with interferon/ribavirin.

#3 Test if new markers associate with interferon/ribavirin treatment emergent suicidal ideation or behavior. Based on published work, interferon/ribavirin treatment increases the risk for suicide and suicidal ideation. Our preliminary studies have also shown that genetic variation may have predictive value in detecting those at risk of developing suicidal ideation, however the described markers have a combined sensitivity of only 88% and a specificity of 99% for citalopram treated patients. This raises the question of whether there are additional markers that have more predictive power and whether depression, suicidal ideation or behavior emergent from IFN/ribavirin treatment is mediated through different pathways and therefore could associate with different markers.

SECTION II. RELATION TO AIMS OF HALT-C STUDY

If specific genetic markers indicating susceptibility to IFN-induced SI and depression can be identified, the ability to recognize individuals for whom IFN/ribavirin treatment may be detrimental or unsuccessful due to psychiatric concerns will increase remarkably. Hence, recognizing these individuals would offer the possibility of closer and more specific follow up or preventative treatment.

SECTION III. BACKGROUND AND SIGNIFICANCE

The combination of pegylated interferon alpha (IFN) and ribavirin, the most widely prescribed and effective treatment for cases of chronic hepatitis C (HCV). IFN is frequently associated with psychiatric side effects including major depression and suicidal ideation (SI), attempts and completed suicide (3-8). HCV, a blood-borne viral disease targeting the liver, affected an estimated 3% of the world's population (300 million individuals) in 2000, with an expected growth rate of 3 to 4 million cases per year (9). The HCV infection rate is particularly high among drug abusers who come into frequent contact with injections (reported between 74% and 100% (10)), and among those diagnosed with a psychiatric disorder (reported between 8% and 19% (11)). Because there is evidence to suggest that HCV patients beginning IFN/ribavirin treatment who have a pre-existing psychiatric condition score higher on measures of depression during treatment than do those patients with no history of psychiatric illness, many gastroenterologists could be reluctant to prescribe the IFN/ribavirin treatment to patients with a known comorbid psychiatric illness. The current pretreatment assessment of risk includes screening HCV patients for psychiatric

disorders and a history of substance abuse, educating them about the treatment process and potential side effects, and evaluating their available psychosocial support (11). If specific genetic markers indicating susceptibility to IFN-induced depression and SI could be identified, the ability to recognize individuals for whom IFN/ribavirin treatment may be detrimental or even fatal will increase remarkably.

Various case studies from Europe and the United States present examples of HCV patients with no history of psychiatric disorder or substance abuse who begin to express SI beginning IFN/ribavirin treatment (3; 7; 8). Although in most cases the indicators of SI disappeared after the completion or early cessation of treatment, there are documented cases of completed suicides attributed to IFN treatment (8). A study of 306 HCV patients found a 1.3% rate of suicide attempts in the six months following the end of treatment (12). Another study evaluated depression in 55 HCV patients both before and during IFN treatment, and found an increase in the percentage of patients with suicidal thoughts from 27% pre-treatment to 43% during treatment (5). While it could be argued that patients with HCV might have significant lifestyle differences that exposes them to increased mental illness including depression and suicide, IFN treatment has also been associated with SI in cases of melanoma (13). The incidence of depression in HCV patients is most frequently attributed to the IFN component of the treatment, although ribavirin has also been linked to depressive symptoms when dosed according to body weight (14). De-novo cases of depression or anxiety disorders occurred in 36% of HCV patients treated (15).

Even though the mechanism by which IFN induces SI remains unknown, it has been hypothesized that the onset of depressive symptoms in HCV patients treated with IFN/ribavirin is linked to a proinflammatory immune response. IFN works by stimulating the activities of various proinflammatory cytokines, including interleukin-6 (IL-6), which promotes increased tryptophan metabolism into quinolinic acid rather than serotonin (16); the resulting deficiency in serotonin is commonly associated with depressive symptoms. The 'low IL-6' synthesizing genotype on the promoter region of the IL-6 gene is thought to partially suppress the immune reaction to IFN and has been associated with decreased risk of depression in IFN treatment (17). A polymorphism on the promoter region of the IFNAR1 gene, which encodes interferon-alpha receptor 1 precursor, has been associated with increased susceptibility to IFN-induced depression (18). In addition to IL-6, other proinflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) typically involved in the immune are response associated with depressive symptoms, but no specific studies have been conducted to attempt to link these genes to an increased risk of SI in HCV patients treated with IFN/ribavirin.

Genetic markers associated with treatment-emergent suicidal ideation (TESI) have been described in patients with MDD treated with citalopram. In a candidate gene study of the STAR*D sample, two markers in genes encoding subunits for ionotropic glutamate receptors associated with TESI. These markers had very high specificity (99%) but low sensitivity (60%) (Laje et al, 2007) (1). Two additional markers were found in the same sample using a genome-wide approach (Laje et al, under review). One of these markers resides in the gene PAPLN, a poorly characterized gene whose encoded protein is present in basal membranes and is expressed in mouse hippocampus (Allen Brain Atlas). The other gene encodes the receptor for IL28A (also known as interferon lambda), part of a newly-discovered set of cytokines that has been associated with interferon alpha (19). These two markers improved sensitivity (88%) but have not yet met the ideal threshold of >95% to potentially translate to clinical use and further replication in independent samples will provide a much better understanding of the true effect of these findings. It remains to be seen if all of these genes have a role in suicidal ideation associated with IFN/ribavirin treatment as well.

There is a significant gap in our understanding of the genetics of depression, suicidal ideation and behavior in the context of drug treatment. Lack of such knowledge is an important problem because detection of variants in these and other genes could help predict the likelihood of emergence of these very serious adverse events. As an outcome of the proposed study, we expect to confirm the interactions between GRIK2, GRIA3, PAPLN, IL28RA and interferon emergent suicidal ideation/behavior and find potentially novel markers with significant predictive power that associate with IFN-emergent depression and SI/behavior. *The proposed research is significant because it is expected to provide knowledge that may ultimately lead to the development of individualized pharmacologic treatment strategies and gain further understanding of effects of long-term interferon/ribavirin treatment.*

SECTION IV. STUDY DESIGN, EXPERIMENTAL GROUPS, METHODS AND DATA USAGE

The proposed study has three specific aims that contribute to our long term goal to understand the underlying pathophysiology of alpha-interferon treatment emergent depressive symptoms and suicidal ideation/behavior and their genetic basis. To pursue this project, we will use DNA samples of participants from the Hepatitis C Antiviral Long-term Treatment Against Cirrhosis (HALT-C) trial and test association of both known markers (specific aim

#2) and genome-wide markers (specific aim #1 and #3) with IFN/ribavirin treatment-emergent suicidal ideation or behavior and development or worsening symptoms of depression.

Sample

Participants were assessed for baseline psychiatric illness using the lifetime & recent depression, anxiety, alcohol and drug use sections from the Composite International Diagnostic Interview (CIDI) (World Health Organization, 2004). The Beck Depression Inventory-II (BDI-II) (20) was implemented to evaluate mood and presence of suicidal intentions at baseline and throughout treatment at weeks 4, 12, 24, 36, 48 and 72 (21).

Power Analysis

For purposes of this power analysis we will use the data parameters associated with the rarer event, treatment-emergent suicidal ideation (TESI). The HALT-C trial has 1013 participants that provided consent for genetic studies, of these 84 subjects met our TESI phenotype definition (8%), leaving 929 controls from the original sample. Power analysis was performed with the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). The number of cases was set at 84 with a control-to-case ratio of 11:1. The high-risk allele frequency was estimated at 0.3, and the marker-disease allele linkage disequilibrium (D') was set to 0.6. Power was greater than 80% to detect association at the p=0.05 level with a variant conferring a heterozygote relative risk of 2.

Phenotype Definition

The phenotype definition for specific aim #1 (onset or worsening of depression > symptoms) will consist of both a continuous and a categorical measure. The continuous measure will be derived from the difference in the BDI-II score from baseline to the highest recorded BDI-II score while taking IFN/ribavirin treatment. Consistent with previous definitions of depression using the BDI (REF, Fontana et al. in press), the categorical phenotype will be defined as:

1. Depression worsening: is defined as an increase in 6 points or more on the BDI-II while receiving IFN/ribavirin, in a patient who had depression at baseline. Depression at baseline is defined as a patient who at baseline had either: 1) a BDI II score ≥ 11 or, 2) fulfilled DSM-IV criteria for major depression during the previous 12 months on the CIDI.
2. Depression onset: Subjects without baseline depression, who experience interferon-induced depression will be defined at any post-baseline time-point as having a BDI ≥ 11 or fulfilling DSM-IV criteria for depression via the CIDI.

Consistent with previous definitions of TESI (1; 2; 21) we will use the suicide item on the self-reported BDI-II scale (item #9) (19). Those individuals who report at baseline (0) "I do not have any thoughts of killing myself," and later in treatment report (1) "I have thoughts of killing myself, but I would not carry them out," (2) "I would like to kill myself," or (3) "I would kill myself if I had the chance" will be considered cases. All others will be considered controls (specific aims #2 and #3).

Exploratory analyses will also be undertaken to identify clinical correlates/ validation of patients with elevated or increasing BDI-II scores or in patients endorsing TESI. Specifically, the indications for pegIFN and ribavirin dose reduction from treatment week 0 to 24 will be reviewed. The BDI-II scores of patients who required a dose reduction/ early discontinuation for neuropsychiatric toxicity will be compared to patients who did not require dose reductions/ early discontinuation. In addition, the frequency of using anti-depressant/ anxiolytic medications at each study visit from treatment weeks 0 to 24 will be determined and the BDI-II scores of patients requiring concomitant psych meds will be compared to those of subjects not requiring adjunct psych meds.

Population Structure

Genetic variation has been introduced to DNA along human evolution; different racial groups carry mutations that may have had evolutionary benefits. As it is possible in case/control studies, differences in allele counts due to racial origin could bias association results. We will evaluate the possibility of population structure leading to inflated association results by using as covariates in the primary allelic test using Eigen-vectors calculated by EIGENSTRAT (21)Additionally, to detect any residual bias we will plot the observed –log10 p-values against the expected –log10 p-values under the null hypothesis (Q-Q plot) and calculate a lambda statistic.

Selection of Single Nucleotide Polymorphism (SNP) Markers and Genotyping Methods

The markers previously described associated with TESI will be genotyped separately using standard TaqMan protocols [GRIA3 (rs4825476), GRIK2 (rs2518224), PAPLN (rs11628713) and IL28RA (rs10903034)]. The Illumina Human610-Quad BeadChip will be used for genome-wide analysis using the Illumina Beadstation 500 according to the manufacturer's recommended protocols. This chip samples over 620,000 SNPs, covering the entire human genome. Based on the over 600k genotypes obtained additional markers will be imputed using in-silico methods to achieve a total of over 2,000,000 known markers reported in HapMap phase 2.

Quality Control

To assure proper analysis of the genotypic data several quality control measures will be taken. Gender mismatched samples will be excluded as well as those with genotyping failures beyond 10%. Markers that deviate from Hardy-Weinberg equilibrium beyond a $1x10^{-3}$ p-value, or have minor allele frequencies below 5% will be excluded from analysis.

Statistical Methods

All association analysis will be carried out using PLINK (22). A logistic regression model will be applied using the Eigen-vectors derived from EIGENTSTRAT as covariates to adjust for potential population stratification that might inflate the association results. Additional covariates derived from univariate analysis of demographic variables and other relevant characteristics will be added to the model as needed. An additive model will be used throughout.

Genome-wide error rate will be controlled by using a standard permutation scheme. This scheme aims at breaking any correlation between the SNP markers and the phenotype, reflecting a global null hypothesis, while maintaining the correlation structure among the markers. By repeating the permutation/analysis steps a large number of times (50,000), the permutation distribution of the minimum p-value will be estimated under the global null hypothesis. Then, by comparing the original raw p-values with the permutation distribution it will allow us to assess their genome-wide significance and compute permutation-adjusted p-values. Additional models, such as genotypic or haplotype tests, will be performed on a post-hoc basis.

Clinical parameters (risk) calculation

The effect of the previously reported markers GRIA3 (rs4825476), GRIK2 (rs2518224), PAPLN (rs11628713) and IL28RA (rs10903034) and the newly found ones will considered as a prospective TESI development. Markers associated with worsening or onset of depressive symptoms will be treated in a similar manner. The risk estimation will be conducted directly on this sample using a standard multivariable logistic regression model. The adequacy of model fit will be assessed via the Hosmer and Lemeshow test (23).

Anticipated problems and alternative strategies

It is possible that despite the relative frequency of depression onset/worsening and suicidal ideation/behavior emerging during interferon treatment our phenotype definitions do not yield enough subjects to successfully test our specific aims. This is a concern particularly for TESI due to its lower frequency, in this case, an expanded phenotype definition will be attempted that will include not only those patients that report "de-novo" suicidal ideation but also those whose suicidal ideation worsens. This implies a higher score on the suicide item of the BDI II at subsequent visits than the one reported at baseline. In the event that some of our aims do not provide any significant results, others could prove to be successful.

If the selected markers, previously described as linked with TESI with citalopram treatment do not show any association signals, this would imply that a different mechanism could be involved in suicidal ideation or behavior secondary to IFN treatment. Finally, if no marker emerges as significant in the genome-wide analysis this would suggest a possible type II error and the top markers would have to be run in an independent sample to look for consistent signals across multiple samples.

SECTION V. ANTICIPATED RESULTS

This project is *innovative* because it combines powerful genetic methodologies to potentially predict emergence of serious side effects. The results could provide new insights into the genetic mechanisms of depression, suicidal ideation and suicide. It is anticipated that this innovative approach will yield the following *expected outcomes*: First, the results will clarify if the relationship between the previously described genes and TESI extends to other drugs. Second, the results will give additional insight into the relationship between genetic markers and IFN/ribavirin treated patients. Overall, the findings will allow us to begin to construct a model of the

relationship between GRIK2, GRIA3, PAPLN, IL28RA and other potentially new genes that will be the basis for future in vitro experiments aimed at defining mechanisms. Our results could also impact the field of pharmacogenetics, advancing the use of genetic markers to match patients with the safest and most effective medication or treatment strategy.

SECTION VI. STATISTICAL SUPPORT

The Genetic Basis of Mood and Anxiety Disorders Unit has all the software, hardware and technical skill and expertise to perform all the analyses proposed. The unit has on staff two full-time data managers for data processing, formatting and querying. The association analysis will be performed using PLINK (see above) on a Unix based platform. This software is currently installed in our servers and available for our use. For those tasks that may require intensive computing capabilities we have both our own dedicated servers and access to the NIH cluster.

NERI will be asked to provide a dataset that will include the following baseline variables:
Age
Gender
Race/ethnicity
Marital status
Education level
Parenteral risk factors (IDU vs blood txf)
Duration of infection
Time since last IFN therapy
Prior IFN vs IFN/ Rib
Serum AST
Platelets
HCV RNA level
HCV genotype
Ishak fibrosis
Diabetes
Hypertension
Lifetime alcohol consumption (Skinner)
CIDI-LT Diagnoses and recency codes
Smoking history

In addition the following on treatment variables will be requested
Virological response at week 12 and week 20
PegIFN dosing through week 24 (% > 80%)
Ribavirin dosing through week 24 (% > 80%)
% with dose reduction and reason at each study visit
% with early discontinuation and reason at each study visit
List of concomitant psychoactive drugs (anti-depressants/ anxiolytics) at each study visit
% with reported suicidal ideation or other psychiatric SAE.

SECTION VII. FINANCIAL ISSUES

Funding for this project will be provided by the Unit on Genetic Basis of Mood and Anxiety Disorders at the Intramural Research Program, National Institute of Mental Health, NIH. UGBMAD funds will cover chips, genotyping, data processing, analysis and UGBMAD staff's time.

SECTION VIII. HALT-C SAMPLES REQUESTED AND DISPOSITION OF DATA

2ug of DNA will be requested from the 1119 patients who entered the lead-in phase of the HALT-C trial and provided genetic consent. All data from the genome wide SNP analysis will be entered into the HALT-C database.

References

1. Laje G eal (2007): Genetic Markers of Suicidal Ideation Emerging During Citalopram Treatment of Major Depression. *Am J Psychiatry* 164.

2. Laje G eal (Genome-wide Association Study of Suicidal Ideation Emerging During Citalopram Treatment of Depressed Outpatients. *Under review* .

3. Ademmer K eal (2001): Suicidal Ideation With IFN-Alpha and Ribavirin in a Patient With Hepatitis C. *Psychosomatics* 42.

4. Bourat L eal (1995): [Attempted suicide during treatment of chronic viral hepatitis C with interferon. Apropos of 2 cases]. *Gastroenterol Clin Biol* 19.

5. Dieperink E eal (2004): Suicidal ideation during interferon-2b and ribavirin treatment of patients with chronic hepatitis C. *General Hospital Psychiatry* 26.

6. Fattovich G eal (1996): A survey of adverse effects in 11,241 patients with chronic viral hepatitis treated with alfa interferon. *J Hepatol* 24.

7. Fukunishi K eal (1998): Burns in a suicide attempt related to psychiatric side effects of interferon. *Burns* 24.

8. Janssen HL eal (1994): Suicide associated with alfa-interferon therapy for chronic viral hepatitis. *J Hepatol* 21.

9. WHO (2007): Hepatitis C.

10. Vignau J eal (2005): Hépatite C, Interféron a et dépression : principales hypothèses physiopathologiques. *Encephale* 31.

11. Rifai MA eal (2006): Psychiatric Management of the Hepatitis C Patient. *Current Treatment Options in Gastroengerology* 9.

12. Rifflet H eal (1998): [Suicidal impulses in patients with chronic viral hepatitis C during or after therapy with interferon alpha]. *Gastroenterol Clin Biol* 22.

13. Windemuth D eal (1999): [Depression and suicidal intentions as a side effect of high dosage interferon-alpha therapy -- two cases]. *Hautarzt* 50.

14. Raison CL eal (2005): Depression During Pegylated Interferon-Alpha Plus Ribavirin Therapy: Prevalence and Prediction. *J Clin Psychiatry* 66.

15. Martín-Santos R eal (2007): De novo depression and anxiety disorders and influence on adherence during peginterferon-alpha-2a and ribavirin treatment in patients with hepatitis C. *Ailment Pharmacol Ther* 27.

16. Müller N and Schwarz MJ (2007): The immune-mediated alteration of serotonin and glutamate: towards an integrated view of depression. *Molecular Psychiatry* 12.

17. Bull SJ eal (2008): Functional polymorphisms in the interleukin-6 and serotonin transporter genes, and depression and fatigue induced by interferon-alpha and ribavirin treatment. *Mol Psychiatry* Epub ahead of print.

18. Yoshida K eal (2005): Promoter Polymorphisms of the Interferon-Alpha Receptor Gene and Development of Interferon-Induced Depressive Symptoms in Patients with Chronic Hepatitis: Preliminary Findings. *Neuropsychobiology* 52.

19. Laje G eal (Genome-wide Association Study of Suicidal Ideation Emerging During Citalopram Treatment of Depressed Outpatients. *Under review* .

20. Beck AT, Steer RA, Ball R, Ranieri W (1996): Comparison of Beck Depression Inventories -IA and -II in psychiatric outpatients. *J Pers Assess* 67: 588-597.

21. Fontana RJ eal (2007): Cognitive Function Does Not Worsen During Pegylated Interferon and Ribavirin Retreatment of Chronic Hepatitis C. *Hepatology* 45.

22. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D et al (2007): PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81: 559-575.

23. Hosmer D., Lemeshow S (2000): *Applied Logistic Regression.* Second ed. New York: Wiley.