APPENDIX A: HALT-C Ancillary Study PROPOSAL

Part I (1 page)
Proposal Name: The Association of Serum Vitamin D Levels and Progression of Hepatic Fibrosis

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HALT-C PI: Jules Dienstag

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

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 (4 page limit, single space)

1. Aims/hypotheses

We hypothesize that lower 25-vitamin D levels are associated with increased rate of fibrosis progression and inflammation. 25-vitamin D has been noted to influence inflammation and fibrosis. 25-vitamin D is formed within the liver and is the predominant form found in the liver. We hypothesize that 25-vitamin D may influence hepatic fibrosis and inflammation and should be measured independently.

2. Background/rationale

Emerging data has demonstrated that Vitamin D plays an important role in a number of biologic processes. Vitamin D is now recognized as an important modulator of both the inflammatory response and of wound healing.(1, 2) Several lines of evidence support the role of vitamin D as an immune modulator through its suppression of TNF-α. In human macrophage cell lines incubated with lipopolysaccharide, treatment with vitamin D resulted in a dose dependent inhibition of TNF-α RNA and protein expression (3, 4). This finding has been replicated in studies of peripheral blood

mononuclear cells.(5, 6) In vitamin D deficient IL-10 knock-out mice a severe, accelerated form of IBD develops. In these mice, treatment with vitamin D results in suppression of several TNF-α related genes, decreased TNF-α production and improvement in colitis.(7)

Additionally, vitamin D deficiency has been shown to play a role in wound healing and the development of fibrosis. Treatment with vitamin D attenuates the development of renal interstitial fibrosis in mouse models by inhibiting renal mRNA expression of fibronectin, types I and III collagen and profibrotic TGF-beta1.(8) Treatment with vitamin D has also been shown to suppress TGF-beta expression in mouse models of diabetic nephropathy.(9)

The importance of vitamin D in immune modulation and fibrosis development may extend to the liver. The liver plays an established role in vitamin D homeostasis. The liver is the site of the conversion of vitamin D-3 to 25-vitamin D and may be a site of vitamin D storage.(10) Both hepatocytes, and to a greater extent, hepatic stellate cells have vitamin D receptors. As in the kidney, Vitamin D is posited to play an anti-inflammatory and anti-fibrotic role in the liver via binding to promotors of target genes leading to downregulation of cytokine and TGF-beta production. Recent work presented at the American Association for the Study of Liver Disease Meeting in 2008 demonstrated that in cells exposed to lipopolysaccharide there was, as expected, a dramatic increase in both neutrophil and monocyte chemoattractants. However, when these same LPS cells were also treated with vitamin D3 there was a dramatic decline in neutrophil and monocytic chemoattractants including Ccl5, Ccl20, Ccrl2, Cxcl5, and CxCl10. Their group posited that vitamin D is able to bind to the promoters of these chemoattractant genes and suppress expression. This group also demonstrated a decrease in the expression of extracellular matrix genes with vitamin D3 treatment mediated by decreased TGF- β production. Further, vitamin D3 treatment decreased production of T macrophage chemotactic protein, TNF-α, TGF-β, IL-6 and IL-1α. (R Evans, unpublished) This data suggests that vitamin D may play a role in both hepatic inflammation and fibrosis.

Cross-sectional data in humans also suggests that vitamin D deficiency is associated with progression of liver disease. Fisher et al. evaluated the vitamin D levels in 100 patients with liver disease, 51 with cirrhosis and 49 without cirrhosis.(11) This group included 38 patients with hepatitis C infection. Ninety-one patients had vitamin D insufficiency (<50nmol/L) which worsened with the progression of liver disease. Chen et al replicated these findings in a cirrhotic cohort noting that vitamin D levels decreased with advancing Childs Class.(12) While an association between vitamin D deficiency and advancing liver disease has been noted, vitamin D deficiency as a predictor for progressive liver disease has not been explored.

3. Relations to aims of HALT-C study

Both our study and the HALT-C study are motivated by a desire to understand and prevent progression of hepatic fibrosis in patients with hepatitis C. HALT-C sought to inhibit fibrosis progression and its complications with pegylated interferon. We seek to determine if vitamin D deficiency enhances fibrosis progression and if so ultimately whether vitamin D supplementation may inhibit fibrosis progression.

4. Study design, experimental groups/5. Methods/Data Usage

We will perform a case control study evaluating the association of study entry serum 25-vitamin D with fibrosis progression. Cases will be defined (as the primary outcome in HALT-C) as patients with Ishak stage 3 or 4 fibrosis (622 patients) with progression of fibrosis over the study period defined by an increase in Ishak fibrosis score or 2 or more stages, patients who suffered death, hepatic decompensation (ascites, encephalopathy, variceal hemorrhage, spontaneous bacterial peritonitis) or Child-Turcotte-Pugh Score of 7 or more on 2 occasions. Patients with HCC as an endpoint will be excluded. Controls will be subjects with stage 3 or 4 fibrosis who did not suffer the primary endpoint at study end (increase in fibrosis stage of 2 or more, death, hepatic decompensation (ascites,

encephalopathy, variceal hemorrhage, spontaneous bacterial peritonitis) or Child-Turcotte-Pugh Score of 7 or more on 2 occasions). Controls and Cases will be matched by age, sex and HALT-C treatment group to cases (134 cases needed).

Using stored sera we will then measure patients baseline/screening serum 25-vitamin D levels to determine the mean levels for both cases and controls.

Inclusion Criteria:

- Liver histopathology available at study entry and study cessation (baseline and 3.5 years)
- Serum available for 25,hydroxyvitamin D
- Ishak fibrosis stage 3 or 4 at study entry

Exclusion Criteria

- Ishak Fibrosis stage 5 or 6 at study entry
- Absence of paired liver histopathology
- Conditions associated with altered vitamin D metabolism or absorption including Intestinal malabsorption, cholestatic liver disease, hyperparathyroidism, end stage renal disease
- Development of hepatocellular carcinoma during the study period

We do not plan to share our data outside of our investigators and study staff.
We plan to publish our results in a peer reviewed journal such as Hepatology or Gastroenterology.

5. Anticipated results

We anticipate that patients with fibrosis progression will have lower levels of 25-vitamin D than patients without fibrosis progression.

6. Statistical support

We plan to evaluate serum 25-vitamin D levels in cases and controls as both continuous variables using Students T test or Wilcoxin rank sum test as appropriate. In addition we evaluate serum 25-vitamin D levels as categorical variables (binary: deficient and normal value as well as in quartiles) using chi square test to create an odd ratio for the progression of fibrosis based on vitamin D levels. We will perform multivariate modeling to evaluate the impact of vitamin D levels, as well as other known variables that influence fibrosis (duration of disease, BMI, EtOH use, DM) on fibrosis progression. Statistical analysis will be performed with SAS software.

With a sample size of 130 subjects per study arm we will have an 80% power to detect a mean difference of 0.3 times the standard deviation of the mean between groups at the 5% level of significance.

Dr. Hui Zheng of the MGH Biostatistics Center will aid in these study.

7. HALT-C samples to be used in the study (complete Part III: Sample Requirements)
8. Financial issues (e.g., cost for data analysis and obtaining samples from Repository)

The procedures of sample collection and preparation will include following steps:

a. Sample selection (by NERI)
   1. Selecting cases according to the criteria described in Section 4.
   2. Matching controls to the cases according to the inclusion/exclusion criteria
b. Serum acquisition (by SeraCare)
   1) Pulling of these samples

2) Sample shipping by FedEx to MGH Labs

c. Vitamin D testing

d. Data analysis

1. Provision of dataset and/or analyses (by NERI or MGH Biostatistics Center).

The cost of selection, shipping, experimentation and data analysis will be assumed by investigator funds.

Using our local statistician and investigators we can perform the data analysis.

9. References

1. Saggese G, Federico G, Balestri M, Toniolo A. Calcitriol inhibits the PHA-induced production of IL-2 and IFN-gamma and the proliferation of human peripheral blood leukocytes while enhancing the surface expression of HLA class II molecules. J Endocrinol Invest. 1989 May;12(5):329-35.
2. Peterlik M, Cross HS. Vitamin D and calcium deficits predispose for multiple chronic diseases. Eur J Clin Invest. 2005 May;35(5):290-304.
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4. Cohen ML, Douvdevani A, Chaimovitz C, Shany S. Regulation of TNF-alpha by 1alpha,25-dihydroxyvitamin D3 in human macrophages from CAPD patients. Kidney Int. 2001 Jan;59(1):69-75.
5. Riancho JA, Zarrabeitia MT, de Francisco AL, Amado JA, Napal J, Arias M, et al. Vitamin D therapy modulates cytokine secretion in patients with renal failure. Nephron. 1993;65(3):364-8.
6. Panichi V, De Pietro S, Andreini B, Bianchi AM, Migliori M, Taccola D, et al. Calcitriol modulates in vivo and in vitro cytokine production: a role for intracellular calcium. Kidney Int. 1998 Nov;54(5):1463-9.
7. Zhu Y, Mahon BD, Froicu M, Cantorna MT. Calcium and 1 alpha,25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease. Eur J Immunol. 2005 Jan;35(1):217-24.
8. Tan X, Li Y, Liu Y. Therapeutic role and potential mechanisms of active Vitamin D in renal interstitial fibrosis. J Steroid Biochem Mol Biol. 2007 Mar;103(3-5):491-6.
9. Zhang Z, Sun L, Wang Y, Ning G, Minto AW, Kong J, et al. Renoprotective role of the vitamin D receptor in diabetic nephropathy. Kidney Int. 2008 Jan;73(2):163-71.
10. Holick MF. Vitamin D deficiency. N Engl J Med. 2007 Jul 19;357(3):266-81.
11. Fisher L, Fisher A. Vitamin D and parathyroid hormone in outpatients with noncholestatic chronic liver disease. Clin Gastroenterol Hepatol. 2007 Apr;5(4):513-20.
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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		268, 0.25 ml of serum			
Lead in Week 4					
Week 8					
Week 12					
W16					
Week 20					
Week 24					
Randomized Month 9		268, 0.25 ml of serum			
Month 12					
Month 15					
Month 18					
Month 21					
Month 24					
Month 27		268, 0.25 ml of serum			
Month 30					
Month 33					
Month 36					
Month 39					
Month 42					
Month 45		268, 0.25 ml of serum			
Month 48					
Post-treatment					
Responders W30					
W36					
W42					
W48					
W60					
W72					

Data needed: We would like to evaluate the 25-vitamin D level (0.25cc of serum) for each subject. This blood would be drawn to correlate with the three liver biopsies received as well as at randomization. To determine our cases versus controls we would need to identify 134 subjects with Ishak stage 3 or 4 at randomization who progressed by 2 or more stages during the course of the study. Controls would also be subjects with Ishak stage 3 or 4 fibrosis at randomization without increase in fibrosis stage at study completion.

25-Vitamin D samples per the MGH and Mayo labs as well as MGH endocrinologists (Marie DeMay) can be stored for 9-10 years without degradation. The Mayo lab has confirmed that vitamin D samples can endure at least 3 freeze/thaw cycles without degradation of the sample.