# A Functional Polymorphism in the Epidermal Growth Factor Gene Independently Predicts Clinical Decompensation in HCV-Related Cirrhosis

# A Functional Polymorphism in the Epidermal Growth Factor Gene Independently Predicts Clinical Decompensation in HCV-Related Cirrhosis

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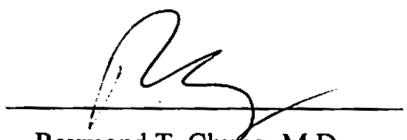
I have reviewed this thesis. It represents work done by the author under my supervision and guidance.



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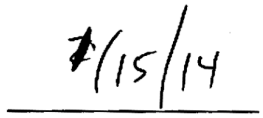

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## ABSTRACT

### Background and Aims

Several single nucleotide polymorphisms (SNPs), including rs4444903 in the epidermal growth factor (*EGF*) gene, rs12979860 near the interleukin-28B (*IL28B*) gene, and rs738409 in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene, have been linked to treatment response, steatosis, fibrosis, and development of hepatocellular carcinoma (HCC) in chronic hepatitis C (HCV). No study has comprehensively examined the effects of these SNPs on the natural history of HCV-related cirrhosis.

### Methods

We performed a retrospective cohort study of 169 subjects with chronic HCV and biopsy-proven cirrhosis who had long term followup for clinical events. Formalin-fixed, paraffin-embedded liver biopsy specimens were genotyped for *EGF*, *IL28B*, and *PNPLA3* using a TaqMan assay with commercial probes and primers. Cox proportional hazards modeling was used to determine the hazard ratio for clinical decompensation, defined as the development of ascites, encephalopathy, variceal hemorrhage, HCC, or cirrhosis-related death.

### Results

During a median followup of 6.6 years, 66 patients (39%) experienced clinical decompensation. On univariate analysis, *EGF* non-A/A, *PNPLA3* non-C/C, and *IL28B* non-C/C genotypes were each associated with increased risk of decompensation. In multivariable Cox regression modeling, *EGF* non-A/A genotype was independently associated with an increased rate of clinical decompensation (HR = 3.00, p = 0.005).

### Conclusions

HCV cirrhotics with the *EGF* A/G and G/G genotypes at rs4444903, a functional polymorphism associated with higher intrahepatic EGF levels, have an increased risk of clinical decompensation. Further study of the predictive value of *EGF* genotyping in patients with earlier stages and other etiologies of liver disease is warranted.

## GLOSSARY OF ABBREVIATIONS

**GWAS** = genome-wide association study

**SNP** = single nucleotide polymorphism

**mRNA** = messenger RNA

**EGF** = epidermal growth factor

**PNPLA3** = patatin-like phospholipase domain-containing protein 3

**IL28B** = interleukin-28 B

**NASH** = Nonalcoholic steatohepatitis

**VLDL** = very low density lipoprotein

**LDL** = low-density lipoprotein

**HCC** = hepatocellular carcinoma

**HCV** = hepatitis C virus

**HR** = hazard ratio

**CI** = confidence interval

**IQR** = interquartile range

**HALT-C** = Hepatitis C Antiviral Long Term Treatment against Cirrhosis

**ALT** = alanine aminotransferase

**AST** = aspartate aminotransferase

**FFPE** = formalin-fixed, paraffin-embedded

# INTRODUCTION

## Personalized Medicine

The concept of "personalized medicine" has been widely discussed, due to recent advances in our understanding of human genetics and the ability to conduct genome-wide association studies (GWAS) for specific diseases. The principal goal of personalized medicine is to identify factors that predict an individual's susceptibility to disease, her likely disease course, or the likelihood she will respond to a specific therapy. This information is then integrated with clinical data to design a targeted, individualized treatment plan. Personalized medicine has the potential to improve health care delivery by providing patients with only those interventions from which they are likely to benefit, thereby increasing effectiveness and decreasing unnecessary diagnostic tests and procedures (reviewed in Whitcomb 2012).

One of the major components of personalized medicine is genetics, which can predict disease severity and response to therapies, making it a useful adjunct to the clinical examination. Specifically, single-nucleotide polymorphisms (SNPs) are variations in single nucleotides that have a variety of effects on gene function. SNPs are relatively easy to genotype because only the evaluation of a single locus is required. SNPs are useful in cancer, where genetic aberrations that cause malignant transformation of cells can be used to design targeted therapies. A number of monoclonal antibody therapies have been successfully developed and applied clinically using this approach.

Application of Personalized Medicine to Chronic Hepatitis C Virus (HCV) Infection

Chronic hepatitis C virus (HCV) infection is a common chronic disease for which SNP genotyping has the potential to improve management. HCV is a single-stranded hepatotropic RNA virus transmitted primarily through blood contact, such as by blood transfusions or intravenous drug use, the latter being the most common mode of transmission in the United States. Vertical and sexual transmission can occur, but both are inefficient. There are six HCV genotypes, each associated with specific geographic areas and ethnic groups. The genotypes vary in their clinical course and treatment response (reviewed in Scheel 2013).

HCV enters hepatocytes through a complex process involving multiple receptors. There, its genome is used directly as messenger RNA (mRNA) for translation of a polyprotein, which is cleaved by host and viral proteases into structural proteins as well as nonstructural proteins involved in replication, the so-called replication complex, which also recruits host cell components. A virally encoded RNA-dependent RNA polymerase replicates the genome, after which new virions are assembled (Major 1997). Host immune responses against HCV cause hepatocyte apoptosis, inflammation and fibrosis. HCV also causes dysregulation of cellular proliferation pathways, predisposing hepatocytes to malignant transformation (Llovet 2008). The rate of progression of chronic HCV infection to cirrhosis is gradual but variable among individuals, likely due to a combination of host genetics, viral genetics, and environmental factors. At least 20% of patients with chronic HCV will develop cirrhosis over a twenty-year time period (National Institutes of Health 1997). Attempts have been made to develop risk scores incorporating clinical variables and genetic data to predict the risk of progression to cirrhosis for individual patients (Huang 2007), but these have not been validated.

In addition, the course of cirrhosis, once established, is unpredictable. In one study, the cumulative 5-year rate of clinical decompensation was 28% in patients with HCV-related cirrhosis, and the average time to the first decompensation event was 29 months (Fattovich 2002). These and other data indicate that most cirrhotics remain compensated and largely asymptomatic for years. Variables previously associated with decompensation include high AST/ALT ratio (Fattovich 2002), higher MELD score, low serum sodium (Volk 2012), presence of varices (Gomez 2013) and obesity (Ioannou 2003). However, these factors have yet to be compiled into a single clinically applicable risk score, and the potential role of genetic information is unclear given the thus far limited data. Furthermore, some of these risk factors were identified in studies that included patients with decompensated cirrhosis, limiting their use for risk stratification in compensated disease. Given the wide variation in clinical outcomes among patients with cirrhosis, predictive information would be valuable to providers, patients, and families. The possibility of tailoring clinical management of chronic HCV to genetic data is exciting, but the discovery of ever-increasing numbers of SNPs associated with liver disease mandates careful and rational selection of polymorphisms that have independent predictive value for relevant clinical outcomes.

Epidermal Growth Factor (EGF)

One of the SNPs known to affect the course of chronic HCV is epidermal growth factor (*EGF*) rs4444903, in which an A → G substitution at position 61 in the 5' untranslated region of the *EGF* gene results in enhanced mRNA stability. EGF is a growth factor that plays numerous roles in cell growth, division, and differentiation, particularly for epithelial cells. The binding of EGF to its receptor, EGFR, leads to activation of pathways involved in proliferation, such as RAS and MAPK. Not surprisingly, therefore, *EGF* and *EGFR* have been implicated in malignant

transformation of many different cell types. *EGFR* overexpression correlates with poor prognosis for a variety of malignancies, including breast, colon, and lung cancers (Nicholson 2001). The G/G genotype at *EGF* rs4444903 was first associated with an increased risk for malignant melanoma compared to the A/G and A/A genotypes (Shahbazi 2002).

In the liver, EGF stimulates hepatocyte proliferation in vitro (Blanc 1992), and *EGF* mRNA levels increase in the early phases of liver regeneration (Mullhaupt 1994). In addition to its pro-growth properties, EGF may also play a role in the establishment of HCV infection. EGFR was recently identified as a host factor involved in HCV infection, and ligands that activate EGFR, especially EGF, increase HCV cellular entry (Lupberger 2011). Thus, the possibility that EGF not only promotes hepatocyte growth but also enables a viral agent that promotes liver cancer merits further study.

On the heels of these studies, there is a growing literature linking the natural history of chronic HCV with *EGF* rs4444903 genotype. One particularly active area is using *EGF* to predict the risk for hepatocellular carcinoma (HCC), a known complication of HCV-related cirrhosis. In two independent retrospective cohorts with HCV-related cirrhosis, Tanabe and colleagues found that genotypes A/G and G/G were associated with a 2.4 and 4.0-fold increased risk for HCC, respectively, compared to the A/A genotype. The other important finding from this study was that intrahepatic mRNA in patients with the G allele had a significantly longer half-life than that from the A allele. Additionally, in cell culture, the number of G alleles correlated with intracellular EGF level; G/G cell lines had the highest levels, while A/A had the lowest levels (Tanabe 2008).

The above findings were corroborated using 816 patients from the Hepatitis C Antiviral Long Term Treatment against Cirrhosis Trial (HALT-C) cohort, a randomized, prospective, multi-center study of 1,050 patients with chronic HCV and bridging fibrosis or cirrhosis (Di Besceglie 2008). Subjects with the G/G genotype had roughly double the risk for HCC compared to those with the A/A genotype. This relationship persisted with a similar odds ratio even after adjusting for clinical variables known to be associated with HCC (Lok 2009), including age, sex, ethnicity, baseline fibrosis score, and smoking status. Interestingly, subjects with the A/G genotype were not at increased risk compared to those with A/A. Patients with HCC had increased serum EGF levels, and this difference was most pronounced for subjects with HCC and the G/G genotype (Abu Dayyeh 2011). The advantages of HALT-C for examining the association between *EGF* rs4444903 and HCC included prospective follow-up, a large, ethnically diverse cohort, and the availability of serum and formalin-fixed tissue for patients in the cohort.

Together, the data from these two studies established A/A as the "favorable" genotype and G/G as the "unfavorable" genotype. Whether A/G fits into one or the other of these categories, or represents intermediate risk, remains unclear. The distribution of the *EGF* polymorphism varies dramatically by race; the A/A genotype is observed in approximately 30% of Caucasians but only 10% of Asians and African Americans. However, the variations in risk for HCC are not simply due to race. Two recent meta-analyses have shown that the association between *EGF* genotype and HCC risk is independent of etiology of cirrhosis and race (Yang 2012, Zhong 2012).

*EGF* status may also affect fibrosis progression. In 645 Caucasian patients with chronic HCV, those with bridging fibrosis or cirrhosis were more likely to have the G/G genotype than patients

with less advanced fibrosis. When patients were stratified by genotype, the mean fibrosis scores were the following: A/A 2.97, A/G 3.16, and G/G 3.52, giving a linear trend for the relationship between *EGF* rs4444903 genotype and fibrosis stage. This relationship was independent of clinical variables known to affect fibrosis progression, namely, age, gender, ethnicity, body mass index, alcohol use, diabetes mellitus, HCV genotype, and presence of HCC. However, it only held for patients younger than 50 years; in patients older than 50 years, *EGF* genotype was not associated with fibrosis score (Falleti 2012).

In summary, EGF is a growth factor with known effects on malignant transformation of multiple cell types. It has been implicated on the molecular level in the establishment of HCV infection, and the rs4444903 SNP has been associated in clinical studies with fibrosis progression and development of HCC in the setting of chronic HCV infection.

Interleukin-28B (IL28B)

Another SNP linked to the course and prognosis of chronic HCV is rs12979860, near the interleukin 28B (*IL28B*) gene. *IL28B* encodes interferon lamba3, one of the type III interferons, cytokines induced by binding of antigens to toll-like receptors (Ank 2006). Following the production of type III interferons, various cellular pathways including JAK/STAT are activated, converging with the type I IFN signal transduction apparatus and resulting in interferon-stimulated gene (ISG) transcription (Uzé 2007). Type III interferons inhibit the replication of hepatitis B and C viruses in vitro, suggesting that they are not specific to particular viruses (Robek 2005). In liver tissue specimens, ISG mRNA expression correlates with HCV RNA presence in cells, suggesting that HCV infection induces ISG expression (Wieland 2013). Type

III interferons also inhibit replication of a number of different tumor cells, including HCC cells, in vitro (Abushahba 2010).

The rs12979860 SNP is located upstream of the *IL28B* gene and can be either a C or T allele. Its precise role in gene regulation has not been elucidated, but the C allele has been associated with lower baseline levels of ISGs in liver tissue as well as a steeper decline in HCV RNA on interferon and ribavirin therapy (Honda 2010; Dill 2011). This suggests that *lower* baseline levels of ISGs are associated with a robust increase in their levels in response to treatment. *IL28B* may regulate the natural killer cell response during acute HCV, thereby affecting the "priming" of the adaptive immune system (Depla 2013). Additionally, one study found that patients not infected with HCV were more likely to have the C/C genotype than patients infected with HCV. It is unclear whether the control and infected groups had similar risk factors for acquisition of HCV, but if so, this could suggest a possible protective role of the C/C genotype against HCV infection (Garcia 2013).

Accordingly, the C/C genotype has been strongly associated with spontaneous clearance of HCV and predicts response to treatment with pegylated interferon and ribavirin. It should be noted that there is significant ethnic variation in both *IL28B* genotype and treatment response; in one large study, 52% of Caucasians but only 21% of African-Americans had the C/C genotype (Thomas 2009). Some investigators have postulated that *IL28B* rs12979860 genotype accounts for approximately half of the variation in treatment response between Caucasians and African-Americans (Ge 2009, Thomas 2009).

Given its known role in inflammation, it is presumed that *IL28B* genotype plays a role in fibrosis progression, but data in this regard have been inconsistent. In one study, patients with HCV-related cirrhosis were more likely to have the T/T genotype than patients with lower-stage fibrosis, independent of relevant clinical factors (Fabris 2011). In another, fibrosis progression in patients with chronic HCV and normal or mildly elevated transaminases was independently predicted by the interaction between *IL28B* T/T genotype and low serum cholesterol (Fabris 2012). However, at least two studies have failed to find any association between fibrosis progression and *IL28B* genotype. In 247 treatment-naïve patients with chronic HCV, predictors of fibrosis progression included older age, male gender, steatosis, and HCV genotype 3, but not genotype at rs12979860 (Marabita 2011). Similarly, in a study of treatment-naïve patients and prior null responders, low platelet count, elevated alkaline phosphatase, and steatosis predicted fibrosis progression, while *IL28B* genotype did not (Noureddin 2013). More studies are needed to resolve this discrepancy.

*IL28B* genotype may affect the risk for complications of HCV. In one study, compared to patients with the C/C genotype, those with the C/T genotype were 2.37 times more likely to develop HCC, while those with the T/T genotype were 4.14 times more likely (Fabris 2011). Importantly, one recent study showed an increased rate of adverse clinical outcomes with the C/C genotype, which had previously been considered "favorable." In a cohort of 1,483 patients, both prior non-responders and treatment naïve, with varying stages of fibrosis, C/C genotype was associated with more severe necroinflammation. Most notably, in the treatment-naïve patients, C/C genotype was associated with higher risk for clinical decompensation compared to C/T or T/T, independent of baseline fibrosis score, albumin level, and bilirubin level (Noureddin 2013).

Overall, it appears that the *IL28B* rs12979860 C/C genotype is associated with a more robust anti-viral inflammatory response in the setting of HCV, leading to higher rates of viral clearance. It remains unresolved whether rs12979860 genotype affects fibrosis progression; it is possible that the effects of *IL28B* on fibrosis are mediated through viral clearance, and that the failure to clear portends progressive liver injury due to an enhanced host inflammatory response. A single large, high-quality study has linked *IL28B* to clinical outcomes, but this has not yet been replicated.

Patatin-Like Phospholipase-3 (PNPLA3)

Another gene of interest in chronic liver disease is patatin-like phospholipase-3 (*PNPLA3*). PNPLA3 breaks down triglycerides within hepatocytes. The SNP rs738409, a C → G substitution near the active site, has been of particular interest in liver disease. The G allele may diminish the triglyceride breakdown function of PNPLA3 (He 2010); however, one animal study suggested that the G allele represents a gain of function, leading to generation of new triglycerides (Kumari 2012).

Given the functional significance of PNPLA3, it is not surprising that the G allele at rs738409 is associated with macrovesicular steatosis. Overexpression of *PNPLA3* with the G allele in mice resulted in liver steatosis, whereas overexpression of the C allele did not (Li 2012). This relationship was also found in humans; in 678 patients from the Nonalcoholic Steatohepatitis (NASH) Clinical Research Network, having the C/G or G/G genotypes was associated with steatosis and lobular inflammation, independent of factors predisposing to fatty liver disease including obesity, diabetes, and the metabolic syndrome (Speliotes 2010).

Approximately half of patients with chronic HCV have significant steatosis, which portends a poorer prognosis, including more rapid progression to cirrhosis (Adinolfi 2001) and diminished responsiveness to interferon therapy (Akuta 2002). This may be because steatosis facilitates HCV replication; HCV virions are assembled through hepatocyte mechanisms that normally secrete very low density lipoprotein (VLDL) particles (Negro 2010). In addition, HCV circulates bound to lipoprotein particles, and the low-density lipoprotein (LDL) receptor on hepatocytes facilitates HCV entry (Agnello 1999). Accordingly, in chronic HCV, rs738409 has been associated with both the presence and severity of steatosis and fibrosis (Trepo 2011, Clark 2012). In one study, the G allele was associated with fibrosis progression independent of ALT level and histologic HCV activity, suggesting that PNPLA3 may activate fibroblasts or other molecular fibrogenic processes (Valenti 2011; reviewed in Dongiovanni 2013).

*PNPLA3* genotype may also affect the risk for HCC. Two studies (Valenti 2011, Corradini 2011) have shown an association between the G/G genotype and HCC; however a recent study failed to confirm this association (Guyot 2013). One recent meta-analysis found that risk for HCC increases with the number of G alleles present (odds ratio 1.77 per G allele, compared to genotype C/C). This relationship existed for both HCV and alcoholic liver disease, although it was stronger in alcoholic liver disease (Trepo 2013). The biological mechanisms for *PNPLA3*'s association with HCC are unclear, but may have to do with acceleration of inflammation and fibrosis, promoting malignant transformation of hepatocytes (Valenti 2013).

## AIMS

Taken together, the above data suggest that specific SNPs on or near *EGF*, *IL28B* and *PNPLA3* could be useful for predicting the natural history of chronic HCV. However, no study that we are aware of has evaluated the influence of *IL28B, EGF,* and *PNPLA3* genotypes on the natural history of HCV-related cirrhosis or examined these three SNPs in the same population. *We therefore sought to evaluate the association between these three SNPs and clinical decompensation in a cohort of patients with HCV-related cirrhosis. In addition, we sought to validate clinical factors associated with decompensation that would be appropriate additions to a genotype-based risk stratification system for chronic liver disease.*

# METHODS

## Patients

The cohort was identified using a database that contains the text of all pathology reports from Massachusetts General Hospital from 1990 to the present. A single-keyword natural language search for all biopsies performed between 1990 and 2007 was performed for the following keywords: hepatitis, HCV, HBV, NAFLD, and NASH. Pathology reports were manually reviewed to identify patients whose biopsies were consistent with HCV-related cirrhosis. This study was approved by the Partners Human Research Committee.

## Inclusion and Exclusion Criteria

A manual chart review by two independent reviewers was performed for subjects who were identified by the pathology database search and whose biopsies were consistent with HCV-related cirrhosis. Inclusion criteria for the study were age ≥18 years at time of the index biopsy, positive HCV antibody or HCV RNA, and presence of cirrhosis on the index biopsy (Ishak stage 5 or 6/6 or Metavir stage 4/4). Exclusion criteria included co-infection with human immunodeficiency virus or hepatitis B virus, prior liver transplantation, or any signs of decompensation prior to or within one month of the index biopsy; signs of decompensation that constituted grounds for exclusion were ascites, variceal hemorrhage, spontaneous bacterial peritonitis, hepatic encephalopathy, or HCC. Patients were also excluded if there were no followup data available after the index biopsy.

Baseline characteristics

We collected the following baseline variables from the electronic medical record: age at the time of biopsy, gender, race, past medical history, body mass index, laboratory values including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, platelet count, creatinine, international normalized ratio (INR), prothrombin time, HCV genotype and HCV RNA, alcohol use, and smoking history. Heavy alcohol use was defined as documentation of alcohol abuse or dependence, a history of substance abuse counseling, or consumption of more than 14 drinks per week by males or 7 drinks per week by females. Diabetes was defined as a history of diabetes mellitus in the medical record or the use of diabetic medications. The number of subjects with incomplete data was 39 for creatinine; 48 for HCV RNA; 22 for HCV Genotype; 13 for smoking status; 13 for ALT, AST, albumin, total bilirubin; 9 for platelets; and 8 for alcohol use.

Followup Data and Outcomes

The followup period for each patient was defined as the date of the index biopsy until the occurrence of the first episode of clinical decompensation, death, loss to followup, or December 31, 2012, whichever came first. Clinical decompensation was defined as the development of ascites, hepatic encephalopathy, variceal hemorrhage, HCC, or liver-related death. Outcomes were identified by manual chart review performed by two independent reviewers. The primary outcome was the time to the first episode of clinical decompensation after the index biopsy.

Genotyping

Five 10μm sections were obtained from each formalin-fixed, paraffin-embedded (FFPE) biopsy block using a microtome cutting device. These sections were immediately placed in a sterile tube

that was DNA and RNA free. DNA was extracted from the sections using the QiaAMP FFPE Tissue Kit (Qiagen Inc, Valencia, CA). The extraction was performed once for each patient, unless insufficient concentrations of DNA were obtained for genotyping, in which case it was repeated. This occurred in less than 10 percent of samples. Genotyping was performed on 5 ng of DNA using the 7900HT Fast Real-Time PCR System with commercial TaqMan SNP Genotyping Assays for *IL28B* rs12979860, *EGF* rs4444903, and *PNPLA3* rs738409 (Life Technologies, Grand Island, NY). Primers and conditions are available upon request. Genotypes were assigned using Sequence Detection System (SDS 2.4) software with manual review by two independent investigators, blinded to subject phenotype.

Statistical analysis

We calculated the person-years of follow-up for each individual from the date of the initial biopsy to the development of the first episode of clinical decompensation, date of death, or the end of follow-up, whichever came first. Kaplan-Meier method was used to analyze the time to clinical decompensation. For each SNP, the favorable genotype was compared to the other two genotypes, which were pooled (i.e. *EGF* A/A was compared to "non-A/A," which included A/G and G/G; *IL28B* C/C was compared to pooled C/T and T/T, and *PNPLA3* C/C was compared to pooled C/G and G/G). The log rank test was used for comparison between genotypes. The crude and adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for the effect of the SNPs on the rate of clinical decompensation were estimated with Cox proportional hazards regression models. We examined each SNP in a univariate model. For multivariable analysis, we first examined each SNP in an age, sex, and race adjusted model. Next, we also examined the association of each SNP in a multivariable model additionally adjusting for established predictors of clinical decompensation including baseline albumin, platelets, and total bilirubin

(D'Amico 2006, Fattovich 2002, Ghany 2010, Ghany 2011). Lastly, we included all 3 SNPS in a combined genotype model in which we compared subjects who had an unfavorable genotype for all 3 SNPs to subjects who had two unfavorable or one or zero unfavorable genotypes.

We performed several sensitivity analyses to strengthen the validity of our findings. We performed an analysis excluding HCC and HCC-related death. Further we performed an analysis excluding the one subject who had an outcome within 6 months of index biopsy. Finally, we performed an analysis excluding subjects who were Child-Pugh Class B or missing a Child-Pugh score because of missing laboratory data. A 2-tailed *P*-value less than .05 was considered statistically significant. SAS (SAS Institute, Cary, NC) version 9.3 was used for statistical analyses.

## RESULTS

Patients

Cohort selection is shown in **Figure 1**. The initial keyword search of the Pathology database yielded over 6,000 patients whose pathology report included one of the searched keywords. After manual review of the pathology reports, 370 patients had pathology reports consistent with a diagnosis of HCV-related cirrhosis. After manual chart review, 150 of these patients were excluded, giving 220 eligible patients. FFPE blocks were not available for 38 patients, and genotyping could not be performed on 13 blocks due to insufficient tissue on the block or insufficient concentrations of DNA after extraction was completed. Thus, the final cohort included 169 patients.

Baseline characteristics

The baseline demographic, clinical and genetic data are shown in **Table 1**. The cohort was predominantly Caucasian (84%) males (74%) with a mean age of 50 ± 9 years. One hundred thirty eight patients were Child-Pugh Class A, and eleven patients were Child-Pugh Class B. The Child-Pugh score could not be calculated for 20 patients because of missing laboratory values. The prevalence of the *EGF, IL28B* and *PNPLA3* genotypes in our cohort was as follows: *EGF* A/A 26.6%, A/G 48.5%, G/G 24.9%; *IL28B* C/C 39.0%, C/T 44.4%, T/T 16.6%, and *PNPLA3* C/C 60.3%, C/G 30.2%, G/G 9.5%.

Follow up and Outcomes

The median length of follow up was 6.6 years (range 0.21-19.5 years). One subject experienced an episode of clinical decompensation 76 days after the index biopsy. Otherwise, all episodes of clinical decompensation occurred at least 6 months after the index biopsy. Of the 169 subjects, 66 (39%) developed at least one episode of clinical decompensation during the follow-up period. There were 75 initial clinical outcomes in these 66 subjects (liver-related death, n=1; ascites, n=26; variceal hemorrhage, n=15; hepatic encephalopathy, n=15; hepatocellular carcinoma, n=18). Of the nine subjects who presented with two primary outcomes simultaneously, seven had ascites and hepatic encephalopathy, one presented with variceal hemorrhage and hepatic encephalopathy, and one subject presented with ascites and variceal hemorrhage. The one subject who experienced liver-related death as a first instance of clinical decompensation died of complications related to portopulmonary hypertension.

Univariate Analysis

On univariate analysis, *EGF* non-A/A genotype was associated with an increased rate of clinical decompensation (unadjusted HR 3.31, 95% CI 1.63-6.71, p=0.0009). Similarly, *IL28B* non-C/C versus C/C genotype (unadjusted HR 1.79, 95% CI 1.05-3.05, p=0.03) and *PNPLA3* non-C/C versus C/C genotype (unadjusted HR 1.80, 95% CI 1.11-2.92, p=0.02) were also each associated with an increased rate of clinical decompensation on univariate analysis. Kaplan-Meier estimates of the time to clinical decompensation stratified by *EGF* genotype showed a significantly increased rate of decompensation in patients with *EGF* non-A/A genotypes compared to A/A genotype (**Figure 2**).

Multivariable Analysis

The results of multivariable analyses are shown in **Table 2**. We first used multivariable models adjusted for age, sex, and race. Compared with A/A genotype, *EGF* non-A/A genotype was associated with an age, sex, and race-adjusted HR of 3.20 (95% CI 1.57-6.52, p=0.001) for clinical decompensation. Compared with C/C genotype, *IL28B* non-C/C genotype was associated with an age, sex, and race-adjusted HR of 1.78 (95% CI 1.03-3.06, p=0.04) for clinical decompensation. Compared with C/C genotype, *PNPLA3* non-C/C genotype was associated with an age, sex, race-adjusted HR of 1.79 (95% CI 1.10-2.90, p=0.02) for clinical decompensation.

To evaluate the specific association of genotype independent of other clinical factors, we used multivariable models with age, race, sex and established predictors of disease progression (baseline albumin, platelet count, and total bilirubin). *EGF* non-A/A genotype remained an independent predictor of an increased rate of clinical decompensation (multivariable-adjusted HR=2.87, 95% CI 1.31-6.25, p=0.008). In contrast, there was no longer a significant association between *IL28B* genotype (multivariable-adjusted HR 1.38, 95% CI 0.71-2.68, p=0.34 for non-C/C compared with C/C) and *PNPLA3* genotype (multivariable-adjusted HR 1.45, 95% CI 0.85-2.47, p=0.17 for non-C/C compared with C/C) and clinical decompensation (**Table 2**).

Three SNP Model

When all three SNPs were analyzed together, the presence of 3 unfavorable genotypes (*EGF* non-A/A, *IL28B* non-C/C, and *PNPLA3* non-C/C, HR 4.03, 95% CI 2.13-7.62, p<0.0001) was associated with a significantly increased risk of clinical decompensation compared to the presence of one or zero unfavorable genotypes. The risk for the presence of 2 unfavorable

genotypes did not reach statistical significance (HR 1.79, 95% CI 0.96-3.35, p=0.07), but the trend did ($P_{trend}$ <0.0001) (**Table 3**).

Sensitivity Analysis

When we excluded a single subject who developed an outcome within 6 months of the index biopsy, the age, sex, and race-adjusted hazard ratios for the association of the SNPs with risk of clinical decompensation were not materially altered. Given the previously described association of *EGF* genotype with HCC (Tanabe 2008), we performed a sensitivity analysis excluding HCC from the primary outcome of clinical decompensation and limiting the outcome to the time to development of ascites, variceal hemorrhage, or hepatic encephalopathy. *EGF* non-A/A genotype remained a significant predictor of an increased rate of clinical decompensation (age, sex, race-adjusted HR=2.93, 95% CI 1.30-6.60, p=0.01). The association between *PNPLA3* genotype and clinical decompensation also persisted (age, sex, race-adjusted HR=1.93, 95% CI 1.10-3.37, p=0.02 for non-C/C compared to C/C). However, the association between *IL28B* genotype and clinical decompensation was no longer significant (age, sex, race-adjusted HR=1.52, 95% CI 0.83-2.79, p=0.18 for non-C/C compared to C/C).

We also conducted analyses restricted to those with Child-Pugh Class A cirrhosis, and found that *EGF* non-A/A genotype was associated with the risk of clinical decompensation in Child-Pugh Class A subjects (age, sex, race-adjusted HR=3.30, 95% CI 1.46-7.35, p=0.004). The significant association between *PNPLA3* genotype and risk of clinical decompensation also persisted (age, sex, race-adjusted HR=2.04, 95% CI 1.16-3.60, p=0.01 for non-C/C compared with C/C). The association between *IL28B* genotype and risk of clinical decompensation did not remain statistically significant when the analysis was restricted to the Child-Pugh Class A subjects (age,

sex, race-adjusted HR=1.81, 95% CI 0.95-3.48, p=0.07 for non-C/C compared with C/C). Our sensitivity analyses show that our results for the *EGF* and *PNPLA3* polymorphisms are consistent when HCC is as excluded as an outcome and when the analysis is restricted to Child-Pugh Class A subjects.

## DISCUSSION

### Summary of Major Findings

While clinical decompensation in cirrhosis is likely due to a multitude of clinical and genetic factors, we have found a novel, strong, and independent association between the SNP rs4444903 in the *EGF* gene and the risk of clinical decompensation in patients with HCV-related cirrhosis. The fact that this association remained even when HCC was removed as part of the primary outcome suggests that our findings are not simply due to the occurrence of HCC, which is already known to be associated with *EGF* genotype.

### Proposed Mechanism for *EGF* and Clinical Decompensation

As the discovery of associations of genetic polymorphisms with human disease increases, the ability to show that specific polymorphisms lead to an alteration in the function or level of the gene product is critical. As detailed above, the literature supports a clear functional relationship between EGF and HCV, from entry of HCV into hepatocytes through HCV-mediated inflammation and fibrosis. Therefore, our finding of the strong association between *EGF* genotype and clinical decompensation suggests a key role for *EGF* in the mediation of chronic HCV-related liver damage.

In the liver, *EGF* is known to be involved in both chronic inflammation and in cancer. These processes are separate but related, supported by several facts: inflammatory cytokines (e.g. IL-1) activate pathways promoting tumor development; some tumors, such as HCC, arise at sites of chronic inflammation; and many cellular pathways promote both inflammation and tumor

development. For example, Elk-1 is a transcription factor activated by EGF signaling that leads to cell growth and differentiation; its downstream effects are instrumental in both cancer and inflammation. Pathways such as Elk-1 could explain how *EGF* is associated with both fibrosis and HCC (reviewed in Kasza 2013). There are likely other EGF-activated pathways that have yet to be elucidated and would be important followup studies to this work.

Hepatic inflammation causes scarring, or fibrosis. It is therefore not surprising that *EGF* genotype is associated with fibrosis progression. However, our findings indicate that *EGF* genotype is important even *after* cirrhosis has been established. We suspect that the explanation for this is that inflammation, fibrosis, and clinical decompensation represent a continuum, even if not perfectly linear. The mechanisms by which EGF promotes inflammation, fibrosis, and cancer are likely the same ones by which it promotes clinical decompensation. Cirrhosis is a useful clinical endpoint, but even once established, inflammation and collagen deposition continue (Planas 2004). Further progression of fibrosis increases resistance in the liver, leading to portal hypertension, and diminishes reserves of normal hepatocytes, resulting in synthetic dysfunction. Our findings suggest that these processes are exacerbated by unfavorable *EGF* genotypes.

These mechanistic explanations point to therapeutic possibilities. Agents that prevent fibrosis or cause its regression are being studied in animals; statins are one particularly exciting possibility (Trebicka 2010). If clinical decompensation is truly along the fibrosis continuum regulated by *EGF*, then anti-fibrotic agents could be beneficial in preventing clinical decompensation. Another theoretical possibility is the use of EGF inhibitors, such as erlotinib, to delay clinical decompensation. This would be interesting from a scientific perspective, but may not be feasible because of the cost and risks of erlotinib therapy. Nonetheless, an approach to the identification

of naturally occurring or already approved products with activity against EGF could yield a more cost-effective approach to slowing fibrosis progression.

Should *IL28B* Genotype Predict Clinical Decompensation?

There is also evidence for a functional association between *IL28B* and HCV. In contrast to data for HCV, *IL28B* genotype does not predict fibrosis or HCC risk in patients with cirrhosis related to hepatitis B (HBV) infection, alcohol or fatty liver disease. In addition, patients with HCV-related cirrhosis are more likely to have the T/T genotype than those with HBV-related cirrhosis (Fabris 2011). Despite the non-specificity of type III interferons, our data suggest a unique interaction with HCV.

While *IL28B* non-C/C genotype was associated with an increased rate of clinical decompensation on univariate analysis and in the age, sex, and race adjusted model, we did not detect an independent association in the multivariable Cox proportional hazards model that included baseline albumin, platelets, and total bilirubin. This was surprising, given a recent study showing an increased rate of clinical decompensation in subjects with the C/C *IL28B* genotype compared to the non-C/C genotype in the HALT-C cohort (Noureddin 2013). The differences in our findings may be due to differences in genotype frequencies between the cohorts: the HALT-C study included only prior non-responders to pegylated interferon and ribavirin, who have a higher prevalence of non-C/C genotypes. As expected, therefore, our cohort had a higher proportion of *IL28B* C/C genotype subjects compared to the HALT-C population (39% versus 10%). Given the association between *IL28B* genotype and clinical decompensation we found in univariate and age, sex, and race adjusted models, it is possible that our study was not powered to detect the difference in rates of clinical decompensation between the two groups in the

multivariable model. It is also possible that there was confounding between *IL28B* genotype and the other SNPs, or that there was correlation between the baseline laboratory values and *IL28B* genotype. It is not clear why we found an association between non-C/C genotype and clinical decompensation on univariate analysis, given that Noureddin and colleagues found C/C to be the unfavorable genotype.

In sum, whether *IL28B* genotype has any predictive value in the course of chronic HCV beyond viral clearance is not clear. This effect might not extend to later stages of chronic HCV infection, however. Further studies are needed to answer this important question.

Should *PNPLA3* Predict Clinical Decompensation?

There was a significant association between the *PNPLA3* genotype and clinical decompensation on univariate analysis and in an age, sex, and race adjusted model that was not found in models adjusting for laboratory predictors of decompensation. While *PNPLA3* genotype has been shown to correlate with histology and fibrosis, it is also possible that once cirrhosis has occurred, its predictive value declines.

Even if *PNPLA3* genotype has limited value in chronic HCV-related cirrhosis, the link between HCV and cellular lipid metabolism warrants further investigation. It is known that HMG-CoA reductase inhibitors (statins) impair HCV viral replication because of the dependence of viral replication on freely available lipids (reviewed in Negro 2010). HCV particles produced under conditions of HMG-CoA reductase inhibition have lower infectivity compared to controls (Peng 2013). It is therefore possible that statins could be effective adjuncts to HCV antiviral treatments.

Value of Risk Stratification for Patients with HCV

There are several reasons that risk stratification of patients with chronic HCV is a worthwhile goal, despite the fact that most patients with chronic HCV do not develop cirrhosis. First, cirrhosis is a major public health problem with substantial morbidity and mortality. Worldwide, there were 1.03 million deaths from cirrhosis and 752,000 deaths from HCC in 2010. In the United States, cirrhosis and chronic liver disease were responsible for over 30,000 deaths, or 1.3% of all deaths, in 2009 (Heron 2012). Focusing our attention on chronic HCV is justified, because HCV is a major contributor to chronic liver disease mortality. In the United States, 26% of cirrhosis deaths and 28% of HCC deaths are caused by HCV (Cowie 2013). There are approximately 2.3 million patients infected with hepatitis C in the United States (Ditah 2013), and chronic HCV is the most common cause of liver-related death and liver transplantation in the United States (Charlton 2001).

The second argument for risk stratification is that patients with HCV-related cirrhosis have a much poorer prognosis than those with less advanced fibrosis, indicating the benefits of identifying and treating patients before the development of cirrhosis. Once cirrhosis is established, patients will remain at risk for hepatocellular carcinoma (HCC) and decompensated cirrhosis, characterized by ascites, variceal hemorrhage or hepatic encephalopathy. After decompensated disease occurs, survival is significantly decreased, from a median of 12 years to 2 years (D'Amico 2006). Survival 1 and 5 years after the first episode of decompensation is approximately 81.8 and 50.8 %, respectively (Planas 2004).

Third, the prevalence of HCV is rising. Possible contributors to this trend include intravenous drug use among younger individuals as well as new diagnoses in older individuals who used

intravenous drugs in the remote past. Recently, the Centers for Disease Control and Prevention recommended birth cohort screening for HCV of all individuals born between 1945-1965 (CDC 2012), a recommendation that was endorsed by the United States Preventive Services Task Force (Moyer 2013). Several studies have reinforced the importance of the CDC recommendations. A recent study of over 200,000 adult primary care patients found that about 17,000 had been tested for anti-HCV antibodies, and three-fourths of patients who were positive were born during 1945-1965 (Yartel 2013). Another study of over 5 million veterans found that 64% born between 1945-1965 had been screened for HCV, of whom 13.5% were positive for anti-HCV antibody and 10.3% had positive HCV RNA (Backus 2013). The presence of advanced fibrosis or cirrhosis at diagnosis of hepatitis C is not an uncommon occurrence. One recent study of over 6,000 patients newly diagnosed with chronic HCV found that 11% had cirrhosis at the time of HCV diagnosis (Moorman 2013).

The rising prevalence of HCV will put an increased burden on gastroenterologists and hepatologists, who may not be able to follow every infected individual closely. Detection of infected patients provides opportunities for treatment, HCC screening, and close follow up to monitor for complications in those with cirrhosis. Risk stratification using genetic and clinical information would allow primary care physicians to follow lower-risk patients, with higher-risk patients seen by a hepatologist frequently. This would ensure the subspecialists, who are already carrying large patient panels even in medically resourced areas, are being used in the most effective and fair way.

A possible counterargument to the above is that newer, more effective treatment regimens for HCV will make the disease burden lower and diminishes the returns of studying risk

stratification. It is true that new therapies are being discovered for hepatitis C at a rapid pace, and the emergence of highly effective, all-oral, interferon-free regimens for hepatitis C is already occurring. Recently presented data revealed that even in patients who failed treatment with protease inhibitors against HCV, newer agents including viral replication complex inhibitors can produce rapid declines in HCV RNA and high rates of continued RNA seronegativity (Lawitz 2013).

However, this picture, while rosy, is misleading. Many individuals will not have access to the new all-oral regimens due to cost. Furthermore, risk for HCC remains even after achievement of SVR. A recently presented meta-analysis found that in 1,001 patients with SVR and bridging fibrosis or cirrhosis, 8.5% developed HCC over a cumulative follow-up time of 8 years, giving a residual annual risk of 1% (van der Meer 2013). Another study found a cumulative incidence of HCC of 12.4% in 7 years of follow up in patients who had had HCV eradicated (Kurosaki 2013). Therefore, the development of a risk stratification tool in the era of curative HCV therapy will be very important for cost-effective management of residual risk of hepatic complications in patients with advanced HCV. This may consist of intensive screening for HCC and varices, as well as implementation of chemopreventive strategies (reviewed in Singh 2014).

## LIMITATIONS

Our study was limited by its retrospective design. The date of diagnosis of cirrhosis was not available, raising the question of whether disease duration prior to the index biopsy affected our results. We attempted to account for this by excluding patients who had evidence of decompensated disease prior to or within one month of the index biopsy. Although we attempted to capture the first episode of decompensation for each patient, it is possible that patients had outside providers who diagnosed these complications at an earlier date, which could have affected our time to decompensation analysis. We were unable to calculate MELD scores due to missing data, including many INR values, as prothrombin times were reported until the mid-1990s. In our multivariable models, we used total bilirubin and platelet levels, laboratory values that have been associated in the literature with higher risk for decompensation (D'Amico 2006, Fattovich 2002, Ghany 2010, Ghany 2011). We also calculated Child-Pugh scores.

In addition, we were limited in statistical power by the number of patients with HCV cirrhosis who had undergone biopsies at our center during the study period. The fact that not all biopsy blocks had sufficient tissue for analysis further limited our sample size, demonstrating the limitations of a single-center study. The generalizability of the study is limited by the predominance of Caucasian males in our cohort and by the fact that the study took place at a tertiary care academic medical center. The latter raises the possibility that the patients in our cohort were sicker or more complicated than cirrhotics seen in the community, which could also have affected our results.

## FUTURE DIRECTIONS

### 186-Gene Signature

Given the association between *EGF* genotype and EGF levels, gene expression levels are also likely to be important in risk stratification of patients with chronic HCV. One way to evaluate expression of many genes is a gene signature, which aggregates the mRNA levels of many informative genes. One such signature, originally developed by Hoshida and colleagues in patients with chronic HCV, consists of 186 genes, including *EGF*. Like SNP genotyping, the gene signature can be successfully evaluated in formalin-fixed liver tissue, allowing for retrospective analysis of patients with long follow-up.

The 186-gene signature was developed in patients with chronic HCV and surgically resected HCC. Initially, 6000 genes were chosen that represented the bulk of variation in gene expression in humans; cross-validation yielded 186 genes associated with survival. There was no association between the gene signature of HCC tissue and clinical outcomes, but in *surrounding non-tumor liver tissue*, the gene signature was associated with overall survival (Hoshida 2008). The 186-gene signature was then applied to patients with chronic HCV and Child-Pugh class A cirrhosis. In this cohort, the unfavorable signature of nonmalignant liver was associated with clinical progression to advanced cirrhosis, development of HCC, and poor survival (Hoshida 2013).

Given these promising results, we have performed gene signature analysis on each of our specimens using Nanostring technology in collaboration with Dr. Hoshida, which permits analysis of much smaller quantities of RNA from tissue blocks or slides. We now have

preliminary data indicating that the gene signature is independently associated with clinical decompensation, but the full analysis is forthcoming and will be published separately.

Other Cohorts

Because this cohort was composed of predominately Caucasian males, and because certain SNP genotypes are known to be associated with race and ethnicity, expanding our work to more diverse cohorts would help validate our findings. In particular, a cohort with a predominance of African-Americans would be valuable, given the difference in genotypes frequencies between Caucasians and African-Americans. Cohorts with a higher percentage of women would also be useful. Given these goals, we plan to obtain data from the HALT-C cohort subset used to examine the effect of *IL28B* genotype on clinical decompensation (Noureddin 2013) to determine whether *EGF* and *PNPLA3* genotypes are predictive of clinical decompensation.

Other Etiologies of Cirrhosis

HCV is only one of many disease processes that can cause liver cirrhosis. As such, we plan to examine the effects of *EGF, IL28B* and *PNPLA3* genotypes on clinical decompensation in other etiologies of cirrhosis. *EGF* has only been studied in the setting of chronic HCV, but it is possible that the mechanisms by which it leads to malignancy and clinical decompensation are common to multiple etiologies of liver disease.

The two other diseases we plan to focus on are chronic hepatitis B infection and NASH/NAFLD. Although our initial keyword search captured these patients, the numbers of each, particularly those with NASH were too small for these to constitute analyzable study arms. One of the reasons for this is that the pathologic diagnosis of NASH has only been applied since the mid-

1990s. Before this time, it was described primarily as "toxic/metabolic," a category that also includes alcoholic liver disease. Without clinical data including alcohol use and body mass index, it is difficult to distinguish between these entities in a retrospective analysis. Collaboration with other institutions may be necessary to compile a sufficiently large cohort.

Another possible approach would be to assemble a cohort of "all comers" with cirrhosis, including rare diagnoses such as autoimmune hepatitis, α-1 antitrypsin deficiency, and others. In a heterogeneous cohort of cirrhotics, an association between *EGF* genotype and clinical decompensation would suggest that EGF's role in progression of chronic liver disease is independent of etiology. However, one of the inherent challenges in such a study is that HCV and alcohol are the two most common etiologies of cirrhosis in the United States, and would together account for the majority of the cohort. It would therefore be unclear whether an association with *EGF* or *IL28B* genotypes was due to a large percentage of patients with HCV cirrhosis. One way around this would be to include equal numbers of patients with each diagnosis, if enough patients with rare diagnoses who had had liver biopsies could be found.

Earlier Stages of Fibrosis

*EGF*, *IL28B*, and *PNPLA3* have been associated with fibrosis progression, although data for *IL28B* is conflicting. However, it is unclear whether they predict eventual clinical decompensation for patients at earlier stages of fibrosis. Answering this question would require a cohort with a long follow up period (on the order of 20 years), which presents a number of challenges. It is an important issue; if *EGF* genotype were found to increase the risk for earlier clinical decompensation in patients with chronic HCV and early-stage fibrosis, then genotyping could be performed early in a patient's course in order to risk stratify patients for aggressive early antiviral therapy.

Prospective Validation of Findings

Risk stratification should be used to alter clinical management. Currently, we are planning a prospective cohort study of *EGF* genotype-based HCC screening of patients with chronic HCV and bridging fibrosis or cirrhosis at our center. Patients will receive a risk score that includes *EGF* rs4444903 genotype and clinical variables known to be associated with HCC risk, including age, gender, smoking status, platelet count, and alkaline phosphatase level. Patients with higher risk scores will be screened for HCC with a gadolinium-enhanced MRI every 6 months, whereas those with lower risk scores will continue to have abdominal ultrasounds every 6 months. Our primary outcome over six years of follow up is the incidence of HCC in each risk group. Secondary outcomes are the quantity of additional imaging and associated cost in the higher risk groups and practitioners' adherence to the new screening protocol. Since hepatologists at our center already order CT or MRI imaging for HCC screening in patients they perceive to be at higher risk, we seek to standardize this practice and correctly identify patients at higher risk for HCC. The overall aim of the study is to detect HCC at an earlier stage, when curative therapy might still be possible in all groups, but the high-risk group is targeted for more sensitive screening based on the increased likelihood of developing HCC.

In addition, given the results of the current study, we are considering a prospective study of HCV cirrhotics at our center in which the intensity of outpatient management will be based on a risk score that includes *EGF* genotype and other clinical variables. For example, patients with the G/G genotype might receive more frequent office visits or a careful search for early ascites or encephalopathy. Such targeted management could reduce costs for low-risk patients and properly intensify management for higher-risk patients.

Other SNPs

Additional SNPs on or near the genes we investigated could be associated with clinical decompensation in chronic HCV. For example, genotype at *IL28B* rs8099917 has been shown to improve prediction of SVR when added to rs12979860 genotype (Fischer 2012). In addition, a dinucleotide variant (deletion of one position and substitution of the other) in the newly described interferon lambda 4 (*IFNL4*) locus predicts SVR as well or better than *IL28B* genotype and is in strong linkage disequilibrium with *IL28B* (Holmes 2013). Finally, a recent whole-exome sequencing study identified a novel locus in the promoter region of *IL28B* that is associated with spontaneous clearance of HCV (Rao 2013). Until clear functional relationships are confirmed, it is possible that already-identified SNPs are simply linked to more important loci on the same or different genes.

SNPs on genes functionally unrelated to *IL28B, EGF*, and *PNPLA3* hold promise as well. One SNP on the *MERTK* gene, which is involved in phagocytosis of apoptotic cells by macrophages, has been associated with higher risk for HCC in patients with HCV who did not respond to interferon and ribavirin (Di Marco 2013). Several SNPs on the *MICA* gene, which is upregulated in tumor cells and in response to many cellular insults, have also been associated with risk for HCC in the setting of HCV cirrhosis (Lo 2013). We plan to additionally genotype SNPs in our cohort that have been shown in the literature to be associated with some aspect of prognosis in chronic HCV.

Liver Transplantation

We also plan to expand this study's aims to liver transplantation. Both donor and recipient *IL28B* genotype have been linked to liver transplant outcomes (reviewed in Duarte-Rojo 2013), including HCV recurrence timing (Duarte-Rojo 2012) and severity (Biggins 2013). Data conflict regarding *IL28B*'s effects on graft fibrosis (Duarte-Rojo 2012, Lange 2011) and graft survival (Duarte-Rojo 2012, Allam 2013). Furthermore, it is unclear which *IL28B* genotype is favorable, and whether it is the same for donors and recipients. In one cohort of 440 recipients, genotypes C/T and T/T were associated with a higher risk of severe recurrent HCV after transplant compared to C/C. However, in a cohort of 225 donor/recipient pairs as part of the same study, C/C donors matched with non-C/C recipients had an increased risk for severe recurrent HCV, an association that was independent of clinical variables known to confer risk. C/C donors matched with C/C recipients showed a trend toward increased risk for recurrent HCV that did not reach significance (Biggins 2013). It appears, therefore, that for recipients, C/C genotype is favorable, whereas for donors, C/C genotype is unfavorable. The reasons for this discrepancy are unclear, but might have to do with C/C promoting HCV eradication in recipients but causing increased levels of inflammation, leading to faster progression to cirrhosis, in the allograft.

Neither *EGF* nor *PNPLA3* has been studied in liver transplantation. Based on non-transplant studies, these SNPs could play an important role in post-transplant outcomes. As such, we are currently undertaking a multi-center, retrospective study of multiple SNPs in HCV donor/recipient pairs with a minimum follow up time of 5 years. The primary outcome is graft survival; secondary outcomes are HCV recurrence, graft cirrhosis, episodes of rejection, and overall mortality.

## IMPLICATIONS AND CONCLUSION

Our work has implications for the application of personalized medicine to clinical practice and to research. First, genetic information is a powerful predictor of disease course and response to therapy, and can be used to individualize management of complex diseases. There are important caveats to this: loci must have a functional relationship and independent predictive value, and clinical context must be taken into account. Second, cellular and molecular targets for therapy are often shared across diseases. EGF exemplifies this principle, given that its over-expression drives malignancies and disease progression in diverse organ systems. Thus, interdisciplinary collaboration with the aim of elucidating common mechanisms is essential. Finally, cost effectiveness should be considered when using genetic data in clinical practice. For example, using gene signatures to profile expression of multiple predictive loci can improve efficiency and abrogate the need for piecemeal genotyping.

In conclusion, our study, the first to examine the effects of *EGF* genotype on clinical outcomes in patients with HCV cirrhosis, has found a novel, strong, and independent association between the SNP rs4444903 in the *EGF* gene and the risk of clinical decompensation. Our findings have important implications for risk stratification of patients with HCV cirrhosis in order to more effectively manage these complicated patients and offer the potential for design of rational therapeutic strategies to prevent fatal complications of HCV-related liver disease.

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## TABLES

*Table 1: Baseline Characteristics of the Cohort (n = 169)*

| Variable | Number of Patients |
|---|---|
| Mean Age, years (SD) | 50 (9) |
| Median Body Mass Index, kg/m$^2$ (IQR)* | 28 (26-32) |
| Male Sex | 125 (74.0%) |
| Race | |
| White | 140 (82.8%) |
| Black | 9 (5.3%) |
| Other | 18 (10.7%) |
| Unknown | 2 (1.2%) |
| Ever smoker | 107 (63.3%) |
| History of heavy alcohol use | 86 (50.9%) |
| Diabetes Mellitus | 22 (13.0%) |
| Genotype* | |
| 1 | 106 (62.7%) |
| 2 | 22 (13.0%) |
| 3 | 12 (7.1%) |
| 4 | 6 (3.6%) |
| 6 | 1 (0.6%) |
| Unknown | 22 (13.0%) |
| HCV RNA>500,000 IU/mL* | 79 (46.7%) |
| Median AST, U/L (IQR) | 96 (67-136) |
| Median ALT, U/L (IQR) | 105 (65-166) |
| Median Albumin, g/dL (IQR) | 3.9 (3.4-4.2) |
| Median Creatinine, mg/dL (IQR)* | 0.9 (0.8-1.0) |
| Median Total Bilirubin, mg/dL (IQR) | 0.7 (0.5-1.0) |
| Median Platelet x1000/mm$^3$ (IQR) | 148 (104-196) |
| Child-Pugh Class | |
| A | 138 (81.7%) |
| B | 11 (6.5%) |
| Unknown | 20 (11.8%) |
| *EGF* genotype | |
| AA | 45 (26.6%) |
| AG | 82 (48.5%) |
| GG | 42 (24.9%) |
| *IL28B* genotype | |
| CC | 66 (39.0%) |
| CT | 75 (44.4%) |
| TT | 28 (16.6%) |
| *PNPLA3* genotype | |
| CC | 102 (60.3%) |
| CG | 51 (30.2%) |
| GG | 16 (9.5%) |

*Missing data: BMI 99 subjects, Creatinine 39 subjects, HCV RNA 48 subjects, genotype 22 subjects

*Table 2: Cox proportional Hazards Model for Clinical Decompensation*

| **Genotype** | **Cases/ Person-Years** | **Age, sex, race-Adjusted Hazard Ratio (95% CI)** | **P value** | **Multivariable Adjusted Hazard Ratio* (95% CI)** | **P value** |
|---|---|---|---|---|---|
| *EGF* A/A | 9/390 | 1.00 | | 1.00 | |
| *EGF* non-A/A | 57/812 | 3.20 (1.57-6.52) | 0.001 | 2.87 (1.31-6.25) | 0.008 |
| *IL28B* C/C | 19/506 | 1.00 | | 1.00 | |
| *IL28B* non-C/C | 47/697 | 1.78 (1.03-3.06) | 0.04 | 1.38 (0.71-2.68) | 0.34 |
| *PNPLA3* C/C | 32/752 | 1.00 | | 1.00 | |
| *PNPLA3* non-C/C | 34/451 | 1.79 (1.10-2.90) | 0.02 | 1.45 (0.85-2.47) | 0.17 |

*Adjusted for age, sex, race, and baseline total bilirubin, albumin, and platelets

*Table 3: Combined genotype model*

| Genotypes | Cases/ Person-Years | Age, sex, race-Adjusted Hazard Ratio (95%CI) | P value* |
|---|---|---|---|
| 0/1 unfavorable genotypes[+] | 18/569 | 1.00 | |
| 2 unfavorable genotypes | 23/424 | 1.79 (0.96-3.35) | 0.07 |
| 3 unfavorable genotypes | 25/209 | 4.03 (2.13-7.62) | <0.0001 |

*$P_{trend}$<0.0001
[+]unfavorable genotypes include *EGF* non-A/A, *IL28B* non-C/C, and *PNPLA3* non-C/C

## FIGURES

*Figure 1: Identification of the Cohort.*

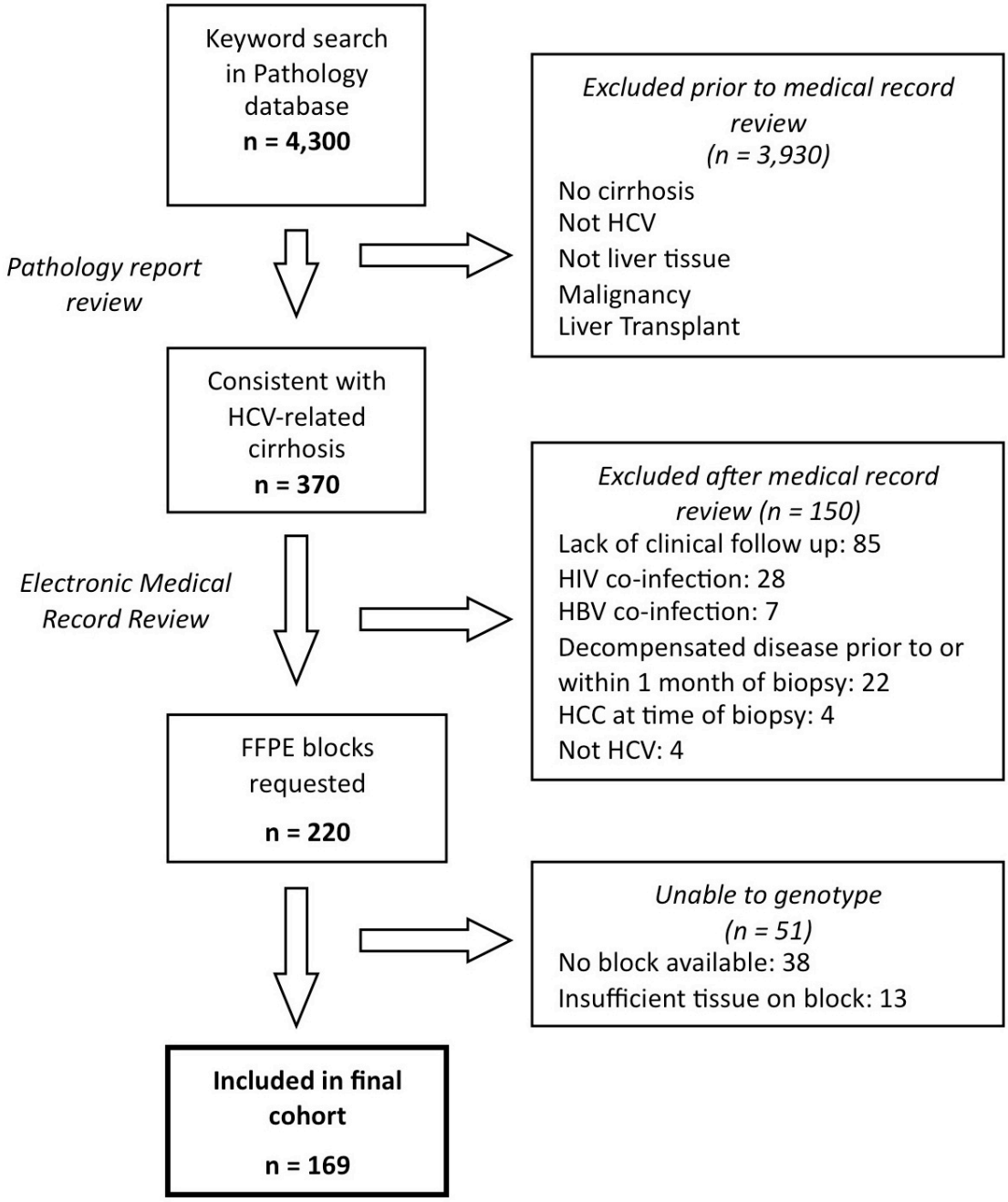

*Figure 2: Time to Clinical Decompensation Stratified by EGF Genotype*