Pre-Transplant Lymphopenia and Early Severe Recurrence of Hepatitis C Disease After Liver Transplantation

A thesis submitted by

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Abstract

Recurrence of hepatitis C viral infection (HCV) after orthotopic liver transplantation (OLT) is nearly universal; however, some patients recur at a much faster rate than others. Low lymphocyte count has been postulated to be a possible surrogate marker for infection risk after liver transplant and for severe early recurrence of HCV after OLT. We hypothesized that pre-transplant lymphopenia is associated with early severe recurrence of hepatitis C after liver transplant. We aimed to evaluate whether pre-transplant lymphopenia was an independent predictor of early severe recurrence of hepatitis C after liver transplant. Retrospective cohort study of 120 liver transplants performed at Tufts Medical Center between 1999-2009. Cox proportional hazards regression analysis was used to examine the association between early severe recurrence of hepatitis C disease, lymphopenia and several other risk factors. The average age of the study population was 51 years, 17% were female. Of these, 25% had a pre-transplant ALC $\leq$ 500/ul and 56% $\leq$1000/ul. Forty two percent of the 120 patients developed fibrosis $\geq$ 2 within 2 years of liver transplant. In univariate analyses, pre-transplant ALC $\leq$500/ul was significantly associated with a reduced rate of early severe recurrence of HCV (HR= 0.41, 95% CI 0.18-0.91). After multivariable adjustment, pre-transplant ALC $\leq$500/ul had a significant protective effect against recurrence (HR 0.40, 95% CI 0.18-0.90). Those transplanted between 1999-2003 (HR = 0.51,95%CI 0.29-0.91) were less likely to have developed early recurrence. Being at increased risk for CMV (CMV IgG recipient or donor positive) was associated with an increased risk of recurrence (HR=2.50, 95%CI 1.16-5.40). Pre-transplant lymphopenia (pre-transplant ALC $\leq$500/ul) was an independent predictor of protection against early severe recurrence of hepatitis C after liver transplant. Low pre-transplant lymphocyte counts may reduce preservation/reperfusion injury which has been associated with progressive fibrosis after transplantation for HCV. Clinicians should be aware that higher pre-transplant lymphocyte counts may result in early HCV related fibrosis and consider such patients for anti-HCV therapy pre or early post-transplant.

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List of Abbreviations

OLT: Orthotopic Liver Transplantation

HCV: Hepatitis C Viral Infection

TMC: Tufts Medical Center

ALC: Absolute Lymphocyte Count

MELD: Model for End Stage Liver Disease

UNOS: United Network of Organ Sharing

MMF: Mycophenolate Mofetil

ATG: Anti-thymocyte globulin

CMV: Cytomegalovirus

CMVIG: Cytomegalovirus Immunoglobulin

HCC: Hepatocellular Carcinoma

CKD: Chronic Kidney Disease

CMV D+/R-: Cytomegaovirus donor IgG positive to recipient IgG negative

CMV D+/R+: Cytomegalovirus donor IgG positive to recipient IgG positive

CMV D-/R-: Cytomegalovirus donor IgG negative to recipient IgG negative

CMV D-/R+: Cytomegalovirus donor IgG negative to recipient IgG positive

Introduction:

Chronic hepatitis C is the most common indication for orthotopic liver transplantation (OLT) among adults. While recurrence of hepatitis C viral infection (HCV) post-liver transplantation is nearly universal, the clinical course of these patients is highly variable. Some patients develop HCV related liver fibrosis slowly (>10 years) while a smaller subset of patients develop fibrosis much more rapidly (within one year).[1, 2] Recurrence of HCV disease is the leading cause of graft failure in patients undergoing liver transplantation and early recurrence, has a significant adverse impact on survival.[2, 3] Several pre- and post-transplant risk factors including advanced donor age and CMV infection have been associated with early recurrence of hepatitis C disease[5-7]; however, there is not one clinical test that predicts recurrence prior to transplantation.

Hepatitis C infection involves an early innate immune response by natural killer cells and dendritic cells as well as a cell-mediated response with viral-specific CD4+ and CD8+ T cells.[8] Patients with end-stage liver disease, regardless of the cause, are known to have decreased peripheral lymphocyte counts as compared with healthy controls.[9] Low lymphocyte count in liver transplant recipients has been evaluated in a small number of studies as a marker for increased risk of infection.[8] Prior studies have found pre-transplant absolute lymphocyte count (ALC) <1000/ul to be significantly associated with the development of a post-transplant infection of any type within 2 years of OLT after multivariable adjustment.[10, 11] Recently, Nagai and colleagues found that pre- and post-liver transplant lymphopenia was associated with higher rates of HCV recurrence with fibrosis stage 2-4 (Metavir scoring) within 2 years of liver transplant on univariate testing. In multivariable survival analysis, post-transplant ALC < 500/uL at 1 month post-OLT remained an independent predictive factor for HCV recurrence. Those patients with persistent lymphopenia during the peri-transplant period (an ALC < 500/ul pre-liver transplant, 2

weeks and 1 month post-transplant) were at significantly increased risk of developing early advanced fibrosis within 2 years.[12]

Understanding that pre-transplant lymphopenia might be a risk factor for early severe recurrence of hepatitis C disease could contribute to the assessment of candidacy for antiviral treatment pre- or post-transplant. We hypothesized that pre-transplant lymphopenia is associated with early severe recurrence of hepatitis C after liver transplant. The aim of this study was to evaluate whether pre-transplant lymphopenia is an independent predictor of early severe recurrence of hepatitis C disease after liver transplantation controlling for several identified confounders. Additionally, as a second aim, we validated previously identified predictors of early severe HCV recurrence in our cohort.

Methods

Study Population and Design:

In this retrospective cohort study, we utilized a liver transplant database that was previously created by the Tufts Medical Center (TMC) Infectious Disease division with the purpose of evaluating the epidemiology, risk factors and clinical presentation of atypical CMV disease in liver transplant recipients. The database captured all liver transplant recipients at TMC from 1999-2009. Data were collected via electronic records as well as paper charts in a systematic fashion. There were 339 liver transplants performed during the study period in 323 patients. The database includes information on patient demographic characteristics as well as baseline or pre-OLT information (patient characteristics, baseline laboratory values), peri-operative information (transfusions, medications, surgical information), and post-transplant information (rejection, treatment of rejection, and infectious outcomes).

In this cohort there were 133 liver transplants in 127 patients with chronic hepatitis C; 6 of these patients underwent re-transplantation. We excluded patients with a first transplant prior

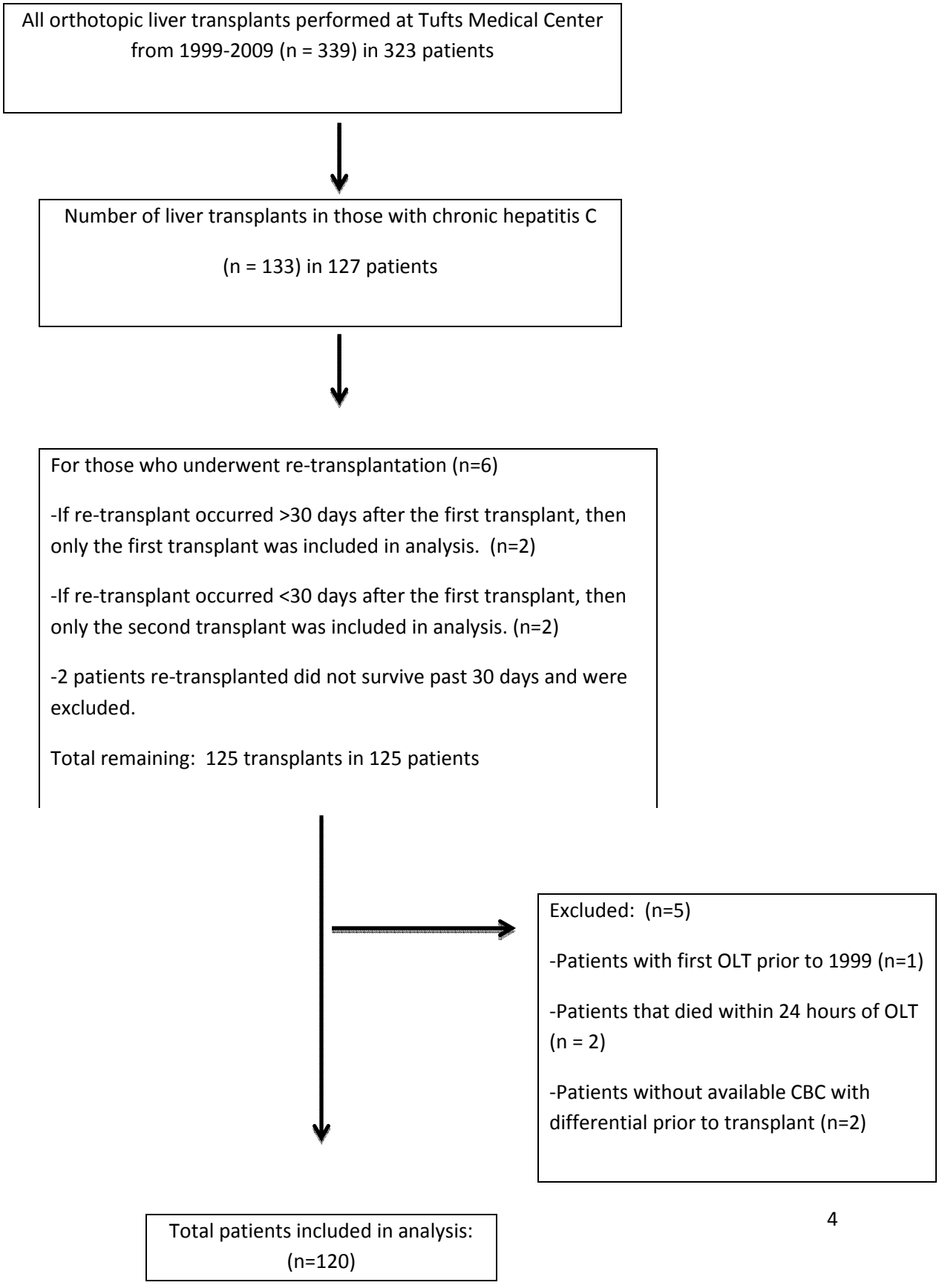
to 1999, patients that died within 24 hours of transplant, patients with incomplete baseline data and patients that had follow up time less than 30 days. Our inclusion/exclusion criteria are described in Figure 1. Two of the 6 patients underwent a second liver transplant greater than 30 days after the first transplant. Only their first transplant episode was evaluated for HCV recurrence. Another two patients had a second transplant less than 10 days after the first transplant and for these we only evaluated the second transplant for HCV recurrence. Baseline variables including pre-transplant ALC, as well as MELD and UNOS status, were taken from prior to the first transplant. The final 2 patients who were re-transplanted did not survive past 30 days and were excluded. There were a total of 120 transplants in 120 patients included in the analysis.

Key Predictor and Outcome Variable:

Pre-transplant lymphopenia was defined and evaluated in two ways: as an absolute lymphocyte count (ALC) of < 1000/ul and $\leq$ 500/ul. Both limits of pre-transplant ALC have been evaluated in prior literature.[10, 12] All patients in our cohort had a complete blood count with differential within 24 hours prior to liver transplantation from which the ALC was calculated.

Our outcome, early severe recurrence of hepatitis C disease post liver transplant, was defined as evidence of chronic HCV disease within 24 months of transplantation on liver biopsy with a fibrosis score of $\geq$ 2 out of 6 on modified Ishak scoring. [4, 6] Protocol liver biopsies were not performed at Tufts Medical Center during our study period. Seventy four patients had liver biopsies during our study period. The decision not to biopsy was a clinical decision made by the transplant and hepatology teams in patients that had no clinical evidence of early recurrence of hepatitis C based on normal liver function tests, clinical stability, and not requiring HCV treatment. Thus, patients without a liver biopsy during the study period were classified as not

Figure 1. Description of inclusion and exclusion criteria for study population



having early severe recurrent HCV. The fibrosis scoring system we used, schema of Ishak, was adopted as the standard histology evaluation schema in 2005 at Tufts Medical Center.[13] The schema of Ishak (or modified Ishak scoring) provides an inflammatory grade (out of 18 points) and a fibrosis stage (out of 6 points). Sixty-two liver biopsies performed during our specified time frame did not provide this specific scoring. A Tufts Medical Center staff pathologist blinded to patient lymphocyte count data and all other clinical data except diagnosis of HCV, reviewed these biopsies and provided modified Ishak scoring.

Additional variable definitions

Several recipient and donor characteristics prior to and at the time of transplant were evaluated for their effect on early severe recurrence of HCV. Post-transplant risk factors of recurrence of HCV were not included in the present analysis. Potential pre-transplant predictors of early severe recurrence of hepatitis C evaluated included demographic data (age at OLT, gender, race), severity of liver disease at time of OLT, transplant year (early or late), comorbidities, donor type (living vs. deceased donor), those with combination liver-kidney transplant and donor/recipient (D/R) CMV serostatus.[1, 5, 6, 14, 15] We also evaluated cold ischemia time and induction immunosuppression at the time of transplantation.[6, 16]

We defined severe liver disease as the designation of Status 1 at the time of OLT, the grading system used until 2000, or subsequently, as having a Model for End Stage Liver Disease (MELD) score of at least 24. We evaluated patients who were CMV D+/-, CMV D+ (and either recipient positive or negative) and patients with either donor or recipient CMV IgG positivity.

Induction immunosuppressive regimen for all patients consisted of cyclosporine or tacrolimus, plus azathioprine or mycophenolate mofetil (MMF) and steroids. Additionally, patients with end stage renal disease received anti-lymphocyte antibodies (ATG) during induction, in lieu of tacrolimus. We evaluated the difference between those receiving MMF and

azathioprine because these agents have different pharmacologic-properties resulting in different immunologic effects; Azathioprine, an antimetabolite, is thought to cause more cytopenias while MMF, a T and B cell inhibitor, is thought to be a more potent immunosuppressive agent. Cyclosporine and tacrolimus are thought to be interchangeable with similar immunomodulatory effects and were not analyzed separately.

We evaluated those patients transplanted between years 1999-2003 compared to those transplanted in 2004-2009 to account for evolution in practice from the earlier transplants compared with those from 2004 onwards. One of these practice changes included a switch from AZA-based immunosuppression to MMF-based regimens. In addition, prior to 2004, standard CMV prophylaxis for high risk patients was IV ganciclovir plus CMV immunoglobulin; starting in 2004 this changed to oral valganciclovir without CMVIG for 90-120 days depending on risk.

Statistical Analysis

Data were summarized, stratified by lymphopenia exposure, using mean with standard deviation for continuous variables and percentage for categorical variables. Student *t* tests, chi-squares and Fisher exact tests were used for two group comparisons. We used Cox proportional hazards regression analysis to analyze the outcome of early severe recurrence of hepatitis C disease. Every analysis began 30 days after liver transplantation since this is the time when it is possible to detect early severe recurrence of hepatitis C disease. Thirty days post transplantation was considered day 0 for the purposes of this study. Patients were censored at 2 years post-OLT, last follow up or death if within 2 years. Kaplan-Meier curves were constructed to estimate the probability of being recurrence-free at 6, 12 and 24 months post-OLT.

Based on prior literature, 30-40% of patients develop early recurrence of HCV within one year of transplantation.[4] Our initial sample size calculation was based on those with an absolute lymphocyte count < 1000/ul, which was 55%. We assumed near equal sample sizes in

each group (lymphopenia yes or no) and estimated that 60% of those with lymphopenia will develop early recurrence of hepatitis C and 30% without lymphopenia develop early recurrence (similar rate as the general HCV liver transplant population). With 80% power and alpha equal to 0.05, 42 patients were needed per group. Our dataset had 120 patients and we assumed adequate statistical power.

Our first aim was to evaluate whether pre-transplant lymphopenia is associated with early severe recurrence of hepatitis C disease following liver transplantation after controlling for potential confounders. In selecting confounding variables, we first used clinical judgment to identify variables that may be associated with both pre-transplant lymphopenia (ALC$\leq$500/ul) and early severe HCV recurrence. We then performed univariate testing of other candidate variables for early severe recurrence of hepatitis C. Those variables, not already evaluated as confounders based on clinical judgment, but with a p value of <0.2 for their univariate association with early severe recurrence of hepatitis C, were also considered for inclusion in the multivariate model. The confounders identified using clinical judgment, which were chronic kidney disease and hepatocellular carcinoma, were forced into the Cox proportional hazards model, and the other candidate variables identified via univariate associations, were evaluated using backward selection. We chose the pre-transplant ALC $\leq$500/ul cut point as our outcome because it had been described in previous literature and because it reflected a more immunocompromised group (compared to those with pre-transplant ALC <1000/ul). The proportional hazards assumption was assessed by examining Schoenfeld residual and log-minus-log survival plots.

Our second aim was to validate previously identified predictors of early severe recurrence of hepatitis C disease in our cohort via univariate testing, as described above. All statistical analysis was performed using R version 3.0.2.

Results

Patient Characteristics

A total of 120 patients comprised our study population. The median age of these patients was 50.8 years, 85% were male and the majority were white (93%). Seventy-five patients (62%) were transplanted between 1999-2003 and the remaining 45 patients were transplanted between 2004-2009. Twenty five percent of patients had a pre-transplant ALC ≤500/ul and 56% of patients had a pre-transplant ALC < 1000/ul. Among our cohort, 9 patients underwent combination liver-kidney transplants and 11 underwent living related donor transplants. Fifty patients (42%) had hepatocellular carcinoma. Thirteen patients (10%) were co-infected with hepatitis B. The majority of patients (82%) received mycophenolate mofetil as induction immunosuppression and a small number of patients (5%) received anti-thymocyte globulin at induction.

Forty two percent of our patient cohort developed the outcome of significant fibrosis (≥2) within 2 years of liver transplant. Of those that recurred, the minimum time to recurrence was 39 days with a median of 199 days and a maximum of 716 days. Estimated probability of being recurrence-free at 6, 12 and 24 months was 78%, 68% and 51% respectively. Recurrence-free survival is displayed by a Kaplan-Meier plot in Figure 2.

Baseline characteristics were assessed by presence of pre-transplant lymphocyte count ≤ 500/ul and displayed in Table 1. Hepatocellular carcinoma (HCC) and chronic kidney disease (CKD) were selected as potential confounders based on clinical grounds. Of note, there was a higher percentage of patients in the non-lymphopenic group with HCC and CKD. There were not any statistically significant differences between those with pre-transplant ALC > 500/ul and ≤ 500/ul, however, a greater proportion of patients with pre-transplant lymphopenia received MMF-based induction immunosuppression (93% vs. 78%).

Figure 2. Kaplan- Meier of HCV Recurrence-Free Survival (N=120)

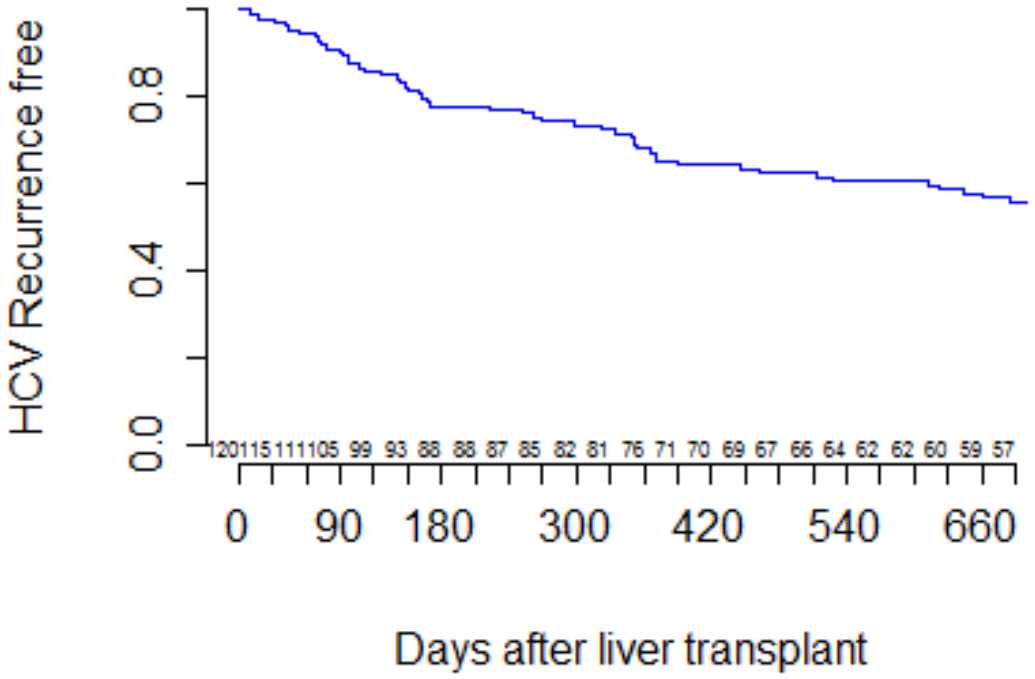


Table 1. Demographic and Clinical Characteristics according to pre-transplant lymphocyte count

	No lymphopenia (ALC> 500 cells/ul) (n=90)	Lymphopenia (ALC≤500 cells/ul) (n=30)	P value
Recipient characteristics			
Transplant age, years, mean (SD)	51 (6.4)	50.6 (6)	0.71
Male gender	74 (82%)	24 (80%)	1.00
Non-white race	8 (9%)	1 (3%)	0.44
Transplant Year, 1999-2003 (vs. 2004-2009)	56 (62%)	19 (63%)	1.00
Severe Liver disease (status 1 or MELD > 24)	49 (54%)	15 (50%)	0.83
Baseline Co-morbidities			
Hepatocellular Carcinoma	40 (44%)	10 (33%)	0.39
Cirrhosis	86 (95%)	29 (97%)	1.00
Alcohol liver disease	35 (39%)	9 (30%)	0.51
Hepatitis B	9 (10%)	4 (13%)	0.73
Diabetes	21 (23%)	5 (17%)	0.61
Chronic kidney disease (GFR<60)	19 (21%)	2 (10%)	0.28
CMV Donor/Recipient serology			
CMV Ig + (recipient or donor)	69 (77%)	20 (67%)	0.40
CMV D+	33 (37%)	14 (47%)	0.45
CMV d+/r-	14 (16%)	7 (23%)	0.49
Cold ischemia time, minutes, mean (SD)	95.9 (53)	84.2 (59)	0.31
Living related donor	9 (10%)	2 (7%)	0.73
Liver-kidney transplant	4 (4%)	4 (13%)	0.11
Induction Immunosuppression			
MMF-based (vs. AZA)	71 (78%)	28 (93%)	0.13
ATG	4 (4%)	2 (7%)	0.63

Univariate analysis

Risk factors for early severe recurrence of HCV were investigated via univariate analysis and displayed in Table 2. On univariate analysis, pre-transplant ALC ≤500/ul was significantly associated with a reduced hazard of early severe recurrence of HCV (HR= 0.41, 95% CI 0.18-0.91, p value= 0.02). Pre-transplant ALC <1000/ul also showed a trend toward a reduced hazard of recurrence (HR 0.70, 95% CI 0.40-1.23), although this was not statistically significant (p =0.22). There were a low number of patients of nonwhite race transplanted in our cohort (n = 9) and hence this variable was not considered for multivariable testing. Patients transplanted prior to 2003 had lower hazard of early severe recurrence of HCV (HR = 0.55, 95% CI 0.31-0.95, p=0.03). Those patients with a history hepatocellular carcinoma had a higher hazard of early severe recurrence however this was not statistically significant (HR 1.6, 95% CI 0.92-2.80, p=0.10). There was not a significant association between severe early HCV recurrence and those patients with who were CMV D+/R- (compared to those CMV D+/R+, CMV D-/R+ and CMV D-/R-) or those CMV D+ (compared to those CMV D-/R-, CMV D-/R+). There was however a significant relationship with those CMV recipient or donor IgG positive (HR=2.18, 95% CI 1.02-4.65, p = 0.04) compared to those CMV D-/R-. There was no significant difference found in early severe HCV recurrence among those receiving mycophenolate mofetil (compared to Azathioprine) or anti-thymocyte globulin (ATG) as induction immunosuppression.

Multivariable Model

Hepatocellular carcinoma and CKD were identified *a priori* as potential confounders and were forced into the multivariable model with pre-transplant ALC ≤500/ul. While performing diagnostics on each candidate variable, the log-minus-log plot suggested CKD may violate the proportional hazards assumption which is displayed by graphs in Figure 3. Therefore this relationship was evaluated using an interaction between CKD and time split at 365 days based on Kaplan-Meier and log-minus-log plots.

Table 2. Univariate risk factors for early severe recurrence of HCV

	No Early Severe Recurrence of HCV (N=70)	Early Severe Recurrence of HCV (N=50)	Hazard Ratio	P value
Recipient Characteristics				
Transplant age, years, mean (SD)	50.4 (6.6)	51.7 (6.1)	1.02 (0.97 -1.07)	0.38
Transplant age >=50	38 (54%)	33 (66%)	1.36 (0.80-2.40)	0.30
Pre-transplant ALC <1000/ul	42 (60%)	25 (50%)	0.70 (0.40-1.23)	0.22
Pre-transplant ALC≤500/ul	23 (32%)	7 (14%)	0.41 (0.18-0.91)	0.02
Male gender	58 (83%)	40 (80%)	0.85 (0.42-1.69)	0.64
Non-white Race	4 (6%)	5 (10%)	2.23 (0.88-5.63)	0.08
Transplant Year, 1999-2003 (vs. 2004-2009)	49 (70%)	26 (52%)	0.55 (0.31-0.95)	0.03
Severe liver disease at txp (status 1 or meld >24)	37 (53%)	27 (54%)	0.97 (0.56-1.70)	0.91
Comorbidities				
Hepatocellular Carcinoma	25 (36%)	25 (50%)	1.60 (0.92-2.80)	0.10
Cirrhosis	65 (93%)	50 (100%)	2.7e+07 (0-inf)	0.99
Diabetes	15 (21%)	11 (22%)	1.12 (0.58 – 2.20)	0.73
Chronic kidney disease (GFR ≤60)	13 (19%)	9 (18%)	0.87 (0.42-1.79)	0.71
Alcohol liver disease	25 (35%)	20 (37%)	1.13 (0.76-2.35)	0.31
Hepatitis B	7 (10%)	6 (12%)	1.16 (0.50-2.73)	0.73
CMV serostatus				
CMV IgG + (recipient or donor)*	47 (67%)	42 (84%)	2.18 (1.02-4.65)	0.04
CMV Donor +/- **	10 (14%)	11 (22%)	1.17 (0.67–2.05)	0.58
CMV Donor + ***	25 (36%)	22 (44%)	1.22 (0.62-2.40)	0.57
Cold ischemia time (>90 min)	35 (50%)	28 (56%)	1.21 (0.69-2.11)	0.51

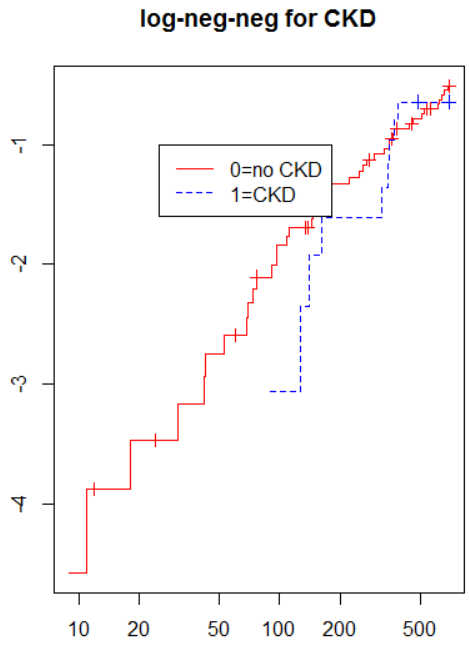
Living related donor	6 (9%)	5 (10%)	1.06 (0.42-2.67)	0.90
Liver-kidney transplant	5 (7%)	3 (6%)	0.87 (0.27-2.80)	0.81
Induction Immunosuppression				
ATG	4 (6%)	2 (4%)	0.70 (0.17-2.90)	0.63
MMF	56 (80%)	43 (86%)	1.48 (0.67-3.30)	0.33

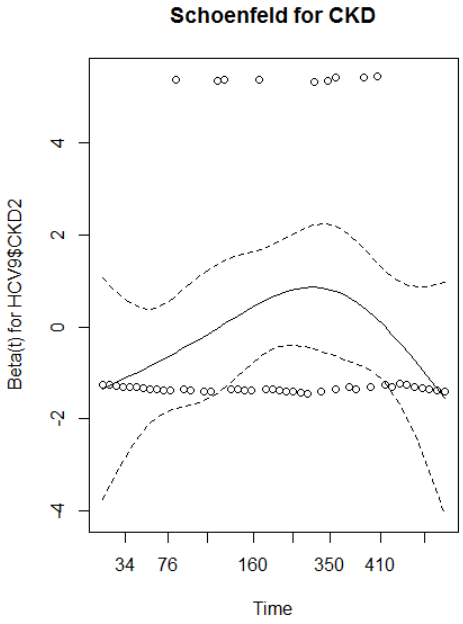
*comparison group: patients CMV D-/R

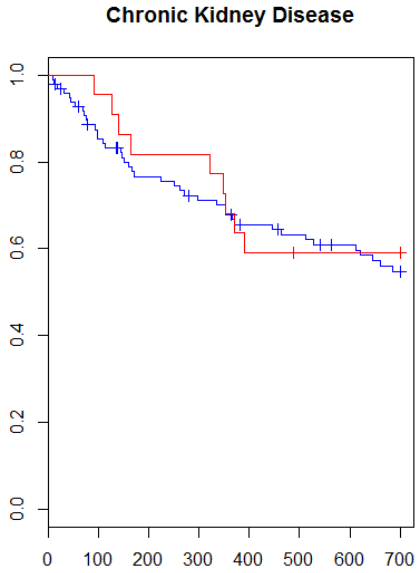
**comparison group: patients CMV D+/R+ or CMV D-/R+ or CMV D-/R-

***comparison group: patients CMV D-/R- or CMV D-/R+

1. **Figure 3.** Kaplan Meier, Schoenfeld residual and log –minus –log plot for chronic kidney disease. These violate the proportional hazards assumption because the two lines cross one another between 300-400 days post OLT. To further evaluate this, we tested the interaction term of prior to 365 days post OLT and after 365 days post OLT (early and late). See below R output. The interaction between CKD and the time period was not significant (p=0.88) suggesting that proportional hazards was not violated.







```
cox23<-
coxph(Surv(HCV10$start,HCV10$Censortime1,HCV10$Recurtot2)~(HCV10$drcmv4+HC
V10$tx9903+HCV10$ALC1cat2+HCV10$HCC+HCV10$CKD2+HCV10$CKD2:HCV10$p
eriod)
summary(cox23)
```

Call:

coxph(formula = Surv(HCV10$start, HCV10$Censortime1, HCV10$Recurtot2) ~

(HCV10$drcmv4 + HCV10$tx9903 + HCV10$ALC1cat2 + HCV10$HCC +

HCV10$CKD2 + HCV10$CKD2:HCV10$period))

n= 195, number of events= 50

	coef	exp(coef)	se(coef)	z	Pr(>|z|)
HCV10$drcmv4	0.9145	2.4956	0.3933	2.325	0.0201
HCV10$tx9903	-0.6642	0.5147	0.2897	-2.293	0.0219
HCV10$ALC1cat2	-0.9118	0.4018	0.4092	-2.228	0.0259
HCV10$HCC	0.4142	1.5131	0.2921	1.418	0.1562
HCV10$CKD2	-0.4913	0.6118	0.4320	-1.137	0.2555
HCV10$CKD2:HCV10$period	-0.1288	0.8791	0.8770	-0.147	0.8832

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

	exp(coef)	exp(-coef)	lower .95	upper .95
HCV10$drcmv4	2.4956	0.4007	1.1545	5.3947
HCV10$tx9903	0.5147	1.9430	0.2917	0.9081
HCV10$ALC1cat2	0.4018	2.4888	0.1802	0.8961
HCV10$HCC	1.5131	0.6609	0.8535	2.6824
HCV10$CKD2	0.6118	1.6345	0.2623	1.4269
HCV10$CKD2:HCV10$period	0.8791	1.1375	0.1576	4.9040

Concordance= 0.685 (se = 0.042)
Rsquare= 0.093 (max possible= 0.899)
Likelihood ratio test= 19.11 on 6 df, p=0.003989
Wald test = 16.75 on 6 df, p=0.01025
Score (logrank) test = 17.17 on 6 df, p=0.008666

The term was not statistically significant (p=0.88) indicating that non-proportionality is likely not a problem, detailed by the R code in Figure 3 above. CKD was therefore included in the model in its original form. Additionally transplant year 1999-2003 and CMV IgG recipient or donor positivity were evaluated using backward selection, after forcing in the confounders. All variables were entered into the final model with multivariate analysis described in Table 3. In the multivariable model, pre-transplant ALC $\leq$500/ul was significantly associated with a reduced hazard of early severe recurrence of HCV (HR 0.40, 95% CI 0.18-0.90, p=0.03). Additionally, CMV recipient or donor IgG positivity remained significantly associated with early severe HCV recurrence (HR=2.50, 95%CI 1.16-5.40, p=0.02) as well as transplant year 1999-2003 (HR = 0.51,95%CI 0.29-0.91, p=0.02). Chronic kidney disease and hepatocellular carcinoma were not significantly associated with early severe recurrence in the multivariable model (HR=0.59, 95%CI 0.28-1.25, p=0.17 and HR =1.52, 95%CI 0.86-2.69, p=0.15, respectively).

Additional analysis

We evaluated those with pre-transplant lymphocyte count <1000/ul, which are displayed in Table 4. There was a larger difference among those with hepatocellular carcinoma (in comparison to those with pre-transplant ALC $\leq$500); 57% in those with pre-transplant ALC $\geq$1000, and 30% in those with pre-transplant ALC <1000. This may be due to the fact that many of these patients were transplanted for hepatocellular carcinoma rather than end-stage liver disease/cirrhosis. More patients receiving a liver-kidney transplant had a pre-transplant ALC <1000/ul however numbers are small (1 and 7 patients). This may be explained by the fact that patients with end stage renal disease have lower levels of peripheral lymphocyte counts.

Table 3. Multivariate adjusted model of Pre-transplant ALC≤500 ul and Early Severe Recurrence of HCV

	Hazard ratio (95% CI)	P value
Pre-transplant ALC ≤500/uL	0.40 (0.18-0.90)	0.03
CMV recipient or donor IgG+	2.50 (1.16-5.4)	0.02
Transplant year, 1999-2003	0.51 (0.29-0.91)	0.02
Chronic kidney disease (GFR ≤60)	0.59 (0.28-1.25)	0.17
Hepatocellular Carcinoma	1.52 (0.86-2.69)	0.15

Table 4. Demographic and Clinical Characteristics by pre-transplant lymphocyte count <1000/ul

	No lymphopenia (ALC≥ 1000/ul) (n=53)	Lymphopenia (ALC<1000 cells/ul) (n=67)	P value
Recipient characteristics			
Transplant age, years, mean (SD)	50.7 (7)	51.1 (5.6)	0.75
Male gender	41 (77%)	57 (85%)	0.40
Non-white race	5 (9%)	4 (6%)	0.51
Transplant Year, 1999-2003 (vs. 2004-2009)	32 (60%)	43 (64%)	0.81
Severe Liver disease (status 1 or MELD > 24)	33 (62%)	31 (46%)	0.12
Baseline Co-morbidities			
Hepatocellular Carcinoma	30 (57%)	20 (30%)	0.01
Cirrhosis	51 (96%)	64 (96%)	1.00
Alcohol liver disease	16 (30%)	28 (42%)	0.26
Hepatitis B	6 (11%)	7 (10%)	1.00
Diabetes	14 (26%)	12 (18%)	0.37
Chronic kidney disease (GFR<60)	13 (25%)	9 (13%)	0.19
CMV Donor/Recipient serology			
CMV Ig + (recipient or donor)	43 (81%)	46 (69%)	0.18
CMV D+	20 (37%)	27 (40%)	0.92
CMV d+/r-	8 (15%)	13 (19%)	0.71
Cold ischemia time, minutes, mean (SD)	101.6 (54.5)	86.1 (54)	0.12
Living related donor	5 (9%)	6 (9%)	1.00
Liver-kidney transplant	1 (2%)	7 (10%)	0.08
Induction Immunosuppression			
MMF-based (vs. AZA)	42 (79%)	57 (85%)	0.55
ATG	1 (2%)	5 (7%)	0.23

We additionally performed sensitivity analyses, examining those patients only who had liver biopsy during our study period (n=74). In Table 5, we performed univariate analysis on groups with liver biopsies only. In Table 6, we performed multivariate analysis on the same variables from our study of the entire cohort. On multivariable adjustment, CKD and HCC appear similar to our model with the entire cohort (N=120). The association of CMV recipient and donor Ig positivity is slightly stronger when analyzing patients with biopsies only (HR 2.5, p 0.01 vs. HR 2.73, p 0.02). Pre-transplant ALC $\leq$500/ul had a weaker protective effect in the group with biopsies only compared to the entire cohort (HR 0.56, p 0.17 vs. HR 0.40, p= 0.03). Additionally transplant year 1999-2003 also had a weaker association with severe HCV recurrence in those only with biopsies (HR 0.77, p 0.40 vs. HR 0.52, p= 0.02).

Those patients that did not undergo liver biopsy during our study period were deemed to not have early severe recurrence of HCV. We compared those patients that did not have a liver biopsy (n=46) to those that did have a liver biopsy but not early severe recurrence of HCV (n=24) via chi- squares or fisher exact tests for dichotomous variables and student t-tests for continuous variables. This is displayed in Table 7. The two groups appeared similar without significant differences. There were slightly more patients without a liver biopsy in those that were transplanted early (1999-2003). Liver biopsies may have been performed more often after 2004 than previously. There was a longer cold ischemia time in those patients with biopsies and no early severe recurrence of HCV, which we cannot explain and is likely random.

Discussion

We found that pre-transplant lymphopenia (ALC $\leq$500/ul) had a significant protective effect against early severe recurrence of hepatitis C after liver transplantation, which is contrary to our hypothesis. The protective effect was observed at both the limit of pre-transplant ALC of $\leq$500/ul and <1000/ul however $\leq$500/ul was more significant. In our cohort, early severe recurrence occurred more often in patients with hepatocellular carcinoma, CMV immunoglobulin

Table 5. Sensitivity analysis of only those patients with liver biopsy (N = 74); Univariate analysis of risk factors for early severe recurrence of HCV for only those patients with liver biopsies

	No Early Severe Recurrence of HCV (N=24)	Early Severe Recurrence of HCV (N=50)	Hazard Ratio	P value
Recipient Characteristics				
Transplant age, years, mean (SD)	50.4 (6.6)	51.7 (5.6)	1.00 (0.94-1.06)	0.90
Transplant age >=50	14 (58%)	33 (66%)	0.96 (0.53-1.73)	0.89
Pre-transplant ALC <1000/ul	12 (50%)	25 (50%)	0.98 (0.57-1.72)	0.97
Pre-transplant ALC≤500/ul	6 (25%)	7 (14%)	0.65 (0.29-1.45)	0.29
Male gender	18 (75%)	40 (31%)	0.99 (0.49-1.98)	0.98
Non-white Race	2 (8%)	5 (10%)	1.57 (0.62-3.97)	0.34
Transplant Year, 1999-2003 (vs. 2004-2009)	13 (54%)	26 (52%)	0.95 (0.54-1.66)	0.86
Severe liver disease at txp (status 1 or meld >24)	14(58%)	27 (54%)	0.85 (0.49-1.50)	0.59
Comorbidities				
Hepatocellular Carcinoma	9 (38%)	25 (50%)	1.28 (0.73-2.23)	0.39
Cirrhosis	21 (88%)	50 (100%)		
Diabetes	5 (21%)	11 (22%)	1.20 (0.61-2.35)	0.59
Chronic kidney disease (GFR ≤60)	5 (21%)	9 (18%)	0.82 (0.40-1.70)	0.60
Alcohol liver disease	6 (25%)	21 (42%)	1.32 (0.75-2.30)	0.33
Hepatitis B	2 (8%)	6 (12%)	1.37 (0.59-3.24)	0.46
CMV serostatus				
CMV IgG + (recipient or donor)*	15 (63%)	42 (84%)	2.26 (1.1-4.80)	0.04
CMV Donor +/- **	5 (21%)	5 (22%)	0.84 (0.43-1.64)	0.60
CMV Donor + ***	9 (38%)	22 (44%)	1.04 (0.59-1.82)	0.89
Cold ischemia time (>90 min)	14 (58%)	28 (56%)	1.02 (0.58-1.78)	0.95
Living related donor	2 (8%)	5 (10%)	1.15 (0.45-2.91)	0.77

Liver-kidney transplant	0	3 (6%)	3.92 (1.17-13)	0.03
Induction Immunosuppression				
ATG	2 (8%)	2 (4%)	0.63 (0.15-2.61)	0.50
MMF	19 (79%)	43 (86%)	1.37 (0.61-3.03)	0.45

*comparison group: patients CMV D-/R

**comparison group: patients CMV D+/R+ or CMV D-/R+ or CMV D-/R-

***comparison group: patients CMV D-/R- or CMV D-/R+

Table 6. Multivariate adjusted model of Early Severe Recurrence of HCV in only patients with liver biopsies

	Hazard ratio (95% CI)	P value
Pre-transplant ALC $\leq$500/uL	0.56 (0.24-1.29)	0.17
CMV recipient or donor IgG+	2.73 (1.24-5.99)	0.01
Transplant year, 1999-2003	0.77 (0.43-1.40)	0.40
Chronic kidney disease (GFR $\leq$60)	0.54 (0.25-1.18)	0.12
Hepatocellular Carcinoma	1.55 (0.87-2.78)	0.14

Table 7. Sensitivity analysis comparing patients with liver biopsies and no HCV recurrence to those patients without liver biopsies.

	Patients without biopsies (n= 46)	Patients with biopsies and no HCV recurrence (n=24)	P value
Recipient characteristics			
Transplant age, years, mean (SD)	50.4 (6.4)	50.4 (5.6)	0.98
Male gender	24 (52%)	14 (58%)	0.81
Non-white race	2 (4%)	2 (8%)	0.60
Transplant Year, 1999-2003 (vs. 2004-2009)	36 (78%)	13 (54%)	0.07
Severe Liver disease (status 1 or MELD > 24)	23 (50%)	14 (58%)	0.68
Pre-transplant ALC <1000/ul	30 (65%)	12 (50%)	0.33
Pre-transplant ALC $\leq$500/ul	17 (37%)	6 (25%)	0.46
Baseline Co-morbidities			
Hepatocellular Carcinoma	16 (35%)	9 (38%)	1.00
Cirrhosis	44 (96%)	21 (88%)	0.44
Alcohol liver disease	17 (37%)	6 (25%)	0.46
Hepatitis B	5 (11%)	2 (8%)	1.00
Diabetes	10 (22%)	5 (21%)	1.00
Chronic kidney disease (GFR<60)	8 (17%)	5 (21%)	0.75
CMV Donor/Recipient serology			
CMV Ig + (recipient or donor)	32 (70%)	15 (63%)	0.74
CMV D+	16 (35%)	9 (38%)	0.98
CMV d+/r-	5 (11%)	5 (21%)	0.29
Cold ischemia time, minutes, mean (SD)	82 (53)	113 (57)	0.03
Living related donor	4 (9%)	2 (8%)	1.00
Liver-kidney transplant	0	5 (11%)	0.16

Induction Immunosuppression			
MMF-based (vs. AZA)	37 (80%)	19 (79%)	1.00
ATG	2 (4%)	2 (8%)	0.60

recipient or donor positive and those transplanted prior to 2004. CMV IgG recipient or donor positivity and transplant prior to 2004 additionally were independent predictors of early severe HCV recurrence. We did not find a difference between those with early severe recurrence in terms of age, gender, other comorbidities, induction immunosuppression and severity of liver disease at the time of transplant. We also did not detect a difference in recurrence among additional risk groups for CMV disease, those CMV D+/R- or CMV donor +.

The one study that had previously evaluated the question or peri-transplant lympopenia and HCV recurrence, found a strong association between post-transplant lymphopenia and HCV disease recurrence, but not with pre-transplant lymphopenia. They found that an ALC of 500-1000/ul and <500/ul one month after transplant were independent risk factors for developing stage 2-4 fibrosis within 2 years of liver transplant. Pre-transplant ALC 500-1000/ul and <500/ul, although had an initial trend toward HCV recurrence (HR 1.41, p =0.06, HR 2.15, p=0.002), was not significantly associated after multivariable adjustment (HR 1.10, p=0.67, HR 1.60, p=0.15, respectively).[12] This is in contrast to our study that found a protective relationship between pre-transplant ALC $\leq$500/ul and early severe recurrence of HCV both on univariate and multivariable testing . Interestingly in their study, induction with rabbit anti-thymocyte globulin (RATG), an agent that reduces peripheral lymphocyte counts, had a protective effect against advanced hepatic fibrosis within 2 years of transplant. The authors postulated that by using RATG induction, they delayed calcineurin inhibitor introduction, which may preserve renal function, reduce the need for peri-transplant dialysis and decrease incidence of rejection in immediate post-transplant period. It is also thought that RATG can alleviate damage caused by ischemia-reperfusion injury, which may also be a potential risk factor for HCV recurrence.[12] A low pre-transplant lymphocyte count may also reduce the risk of ischemia-reperfusion injury which may in part explain our study findings.

We additionally found that those at risk for CMV disease, by being either donor or recipient CMV serology positive, had double the hazard of having early severe recurrence of hepatitis C

compared to those D-/R-. CMV infection and disease has been associated with recurrence of hepatitis C disease within one year of liver transplant.[17, 18] Those patients at highest risk of developing CMV infection or disease are those that are donor CMV IgG+/ recipient CMV IgG- (CMV D+/R-), followed by those CMV D+/R+, then those CMV D-/R+. In a short term model for HCV recurrence, CMV recipient serostatus was found to be predictive of early severe recurrence of HCV.[19] CMV serostatus can be measured pre-transplant and be included in the evaluation for hepatitis C treatment.

In our study, patients transplanted from 1999-2003 were less likely to have early severe recurrence compared to those transplant during years 2004-2009. In the literature, transplants done prior to 2000 were found to be associated with increased HCV-related disease progression.[7, 20, 21] The possible reasons for recent increased HCV disease progression after transplant are increasing age of the donor and use of more potent immunosuppressive drugs. Our findings may be explained by the fact that at our institution, there was more MMF induction therapy and a different CMV prophylaxis strategy starting in 2004.

Hepatitis C infection is associated with a CD8+/interferon-gamma response, followed by a CD4+specific response and antibody production. Hepatitis C disease post-liver transplantation occurs in two forms: (1) severe cholestatic recurrence with extreme viral burden resulting in direct and severe injury to hepatocytes with advanced injury by 6 months post-transplant and (2) progression to chronic hepatitis. This occurs by CD4+ and CD8+ cell-mediated injury to hepatocytes rather than direct injury from the hepatitis C virus. This is more severe in those undergoing liver transplant than immunocompetent individuals. Studies have examined intrahepatic CD4-specific responses and found that HCV-specific interferon-gamma responses can be detected in some patients and may correlate with liver injury.[22] This suggests that for those developing chronic hepatitis C after liver transplant, a strong immune response is occurring,

damaging hepatocytes. One could speculate that those with higher lymphocyte counts pre-transplant may be at greater risk of initial liver injury post-transplant.

Additionally, our finding of early severe recurrence of HCV among those with high pre-transplant lymphocyte counts may be explained in part by more tissue reperfusion injury in these patients. Reperfusion injury occurs after blood flow is restored in graft liver in the recipient. There is cell death from cytokine release (TNF-alpha) and reactive oxygen intermediates that facilitate an inflammatory response. It is associated with hepatocellular death, followed by cellular proliferation. Those patients with preservation injury, or injury from the organ harvesting process and reperfusion injury, after transplantation for hepatitis C, have been shown to have poorer outcomes. Watt and colleagues evaluated the effect of preservation injury (PI) on HCV recurrence post-transplantation by matching those transplanted with HCV to those transplanted without HCV and those with HCV but no preservation injury. They found that among those patients with HCV, those with preservation injury had more progression to stage 3 or 4 fibrosis compared to those without PI. Additionally they had lower one and three year survival rates.[23]

Our study has several important limitations. It was conducted with patients from a single transplant center and was retrospective in design. Our small sample size limited our ability to fully evaluate the relationship between lymphopenia with many covariates in a single model. Notably, our finding of the protective effect of pre-transplant lymphopenia and HCV recurrence, contrary to our hypothesis, may be due to the fact that our small sample size limited our ability to fully evaluate this relationship as well as the relationship between lymphopenia with many covariates in a single model. The surgeons and hepatologists caring for the patients did not employ protocol liver biopsies during our study period. Biopsies were performed during our study period in most patients with persistently elevated liver function tests in order to rule out rejection or recurrent hepatitis C. It is possible however, that a patient with normal liver enzymes who was not biopsied did develop unrecognized stage 2 fibrosis within two years of liver transplant, so there may have been some classification bias introduced. Liver function testing has

not been shown to be a consistent indicator of ongoing liver injury in patients with hepatitis C. We additionally did not have T cell subsets to analyze to see the effect of CD4 or CD8 counts on HCV recurrence, nor the recent gene locus associated with severe hepatitis C disease, IL-28B. We also did not have donor age available, which is a strong predictor of early severe recurrence of hepatitis C. Additionally we did not evaluate post-transplant risk factors such as maintenance immunosuppression, rejection treatment, CMV disease and other post-transplant infections. Evaluating these variables and post-transplant lymphocyte count would be an important analysis which we plan to do in the future on this data set.

Conclusion

In conclusion, pre-transplant lymphopenia (pre-transplant ALC $\leq$ 500/ul) was associated with a reduced rate of early severe recurrence of hepatitis C disease after liver transplant. Although pre-transplant lymphopenia has been shown to be a risk factor for other types of post-transplant infection, this pattern was not observed in the case of post-transplant hepatitis C. Low pre-transplant lymphocyte counts may reduce preservation injury (reperfusion injury) which has been associated with progressive fibrosis after liver transplantation in patients with hepatitis C. Clinicians should be aware that higher lymphocyte counts prior to transplant may result in earlier HCV related fibrosis post-transplant and these patients should be evaluated for anti-HCV treatment pre- or early post-transplant.

References

1. Berenguer M. Natural history of recurrent hepatitis C. - Liver Transpl.2002 Oct;8(10 Suppl 1):S14-8., (1527-6465 (Print); 1527-6465 (Linking)).

2. Gane EJ. The natural history of recurrent hepatitis C and what influences this. - Liver Transpl.2008 Oct;14 Suppl 2:S36-44.doi: 10.1002/lt.21646., (1527-6473 (Electronic); 1527-6465 (Linking)).

3. Prieto M, FAU - Berenguer M, Berenguer M, et al. High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: Relationship with rejection episodes. - Hepatology.1999 Jan;29(1):250-6., (0270-9139 (Print); 0270-9139 (Linking)).

4. Gallegos-Orozco JF, FAU - Yosephy A, Yosephy A, et al. Natural history of post-liver transplantation hepatitis C: A review of factors that may influence its course. - Liver Transpl.2009 Dec;15(12):1872-81.doi: 10.1002/lt.21954., (1527-6473 (Electronic); 1527-6465 (Linking)).

5. Bosch W, FAU - Heckman MG, Heckman MG, et al. Association of cytomegalovirus infection and disease with recurrent hepatitis C after liver transplantation. - Transplantation.2012 Apr 15;93(7):723-8.doi: 10.1097/TP.0b013e3182472876., (1534-6080 (Electronic); 0041-1337 (Linking)).

6. Wiesner RH, FAU - Sorrell M, Sorrell M, FAU - Villamil F, Villamil F. Report of the first international liver transplantation society expert panel consensus conference on liver transplantation and hepatitis C. - Liver Transpl.2003 Nov;9(11):S1-9., (1527-6465 (Print); 1527-6465 (Linking)).

7. Berenguer M, FAU - Prieto M, Prieto M, et al. Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. - Hepatology.2002 Jul;36(1):202-10., (0270-9139 (Print); 0270-9139 (Linking)).

8. Rosen HR. Hepatitis C pathogenesis: Mechanisms of viral clearance and liver injury. Liver Transpl, **2003**; 9(11): S35-43.

9. Romo EM, Munoz-Robles JA, Castillo-Rama M, et al. Peripheral blood lymphocyte populations in end-stage liver diseases. J Clin Gastroenterol, **2007**; 41(7): 713-21.

10. Fernandez-Ruiz M, FAU - Lopez-Medrano F, Lopez-Medrano F, et al. Pretransplant lymphocyte count predicts the incidence of infection during the first two years after liver transplantation. - Liver Transpl.2009 Oct;15(10):1209-16.doi: 10.1002/lt.21833., (1527-6473 (Electronic); 1527-6465 (Linking)).

11. Nierenberg N, Poutsiaka D, Price LL, et al. Baseline lymphopenia as a predictor of serious invasive infections following . In: 2012:.

12. Nagai S, Yoshida A, Kohno K, et al. Peritransplant absolute lymphocyte count as a predictive factor for advanced recurrence of hepatitis C after liver transplantation. Hepatology, **2014**; 59(1): 35-45.

13. Ishak K, FAU - Baptista A, Baptista A, et al. Histological grading and staging of chronic hepatitis. - J Hepatol.1995 Jun;22(6):696-9., (0168-8278 (Print); 0168-8278 (Linking)).

14. Belli LS, FAU - Burroughs AK, Burroughs AK, et al. Liver transplantation for HCV cirrhosis: Improved survival in recent years and increased severity of recurrent disease in female recipients: Results of a long term retrospective study. - Liver Transpl.2007 May;13(5):733-40., (1527-6465 (Print); 1527-6465 (Linking)).

15. Jain A, FAU - Singhal A, Singhal A, et al. Comparative analysis of hepatitis C recurrence and fibrosis progression between deceased-donor and living-donor liver transplantation: 8-year longitudinal follow-up. - Transplantation.2011 Aug 27;92(4):453-60.doi: 10.1097/TP.0b013e3182259282., (1534-6080 (Electronic); 0041-1337 (Linking)).

16. Samonakis DN, FAU - Triantos CK, Triantos CK, et al. Immunosuppression and donor age with respect to severity of HCV recurrence after liver transplantation. - Liver Transpl.2005 Apr;11(4):386-95., (1527-6465 (Print); 1527-6465 (Linking)).

17. Burak KW, Kremers WK, Batts KP, et al. Impact of cytomegalovirus infection, year of transplantation, and donor age on outcomes after liver transplantation for hepatitis C. Liver Transpl, **2002**; 8(4): 362-9.

18. Botha JF, FAU - Thompson E, Thompson E, et al. Mild donor liver steatosis has no impact on hepatitis C virus fibrosis progression following liver transplantation. - Liver Int.2007 Aug;27(6):758-63., (1478-3223 (Print); 1478-3223 (Linking)).

19. Charlton M, Ruppert K, Belle SH, et al. Long-term results and modeling to predict outcomes in recipients with HCV infection: Results of the NIDDK liver transplantation database. Liver Transpl, **2004**; 10(9): 1120-30.

20. Berenguer M, Crippin J, Gish R, et al. A model to predict severe HCV-related disease following liver transplantation. Hepatology, **2003**; 38: 34-41.

21. Berenguer M, Ferrell L, Watson J, et al. HCV-related fibrosis progression following liver transplantation: Increase in recent years. J Hepatol, **2000**; 32(4): 673-84.

22. McCaughan GW, Zekry A. Pathogenesis of hepatitis C virus recurrence in the liver allograft. Liver Transpl, **2002**; 8(10 Suppl 1): S7-S13.

23. Watt KD, Lyden ER, Gulizia JM, McCashland TM. Recurrent hepatitis C posttransplant: Early preservation injury may predict poor outcome. Liver Transpl, **2006**; 12(1): 134-9.