Anca Zimmermann¹, Felix Darstein¹,², Maria Hoppe-Lotichius³, Gerrit Toenges¹, Anja Lautem³, Frédéric Abel¹, Arno Schad³, Jens Mittler²,³, Johanna Vollmar¹, Daniel Grimm¹, Hauke Lang²,³, Peter R. Galle¹,², Tim Zimmermann¹,² *, Detlef Schuppan⁶,⁷ *

INTRODUCTION

Liver transplantation (LT) is the last therapeutic option for patients with end-stage liver disease. The development of LT into a successful therapy over the last few decades has resulted in improved postoperative short- and long-term survival. Therefore, management of comorbidities and prevention of recurrence of the underlying disease has become increasingly important, especially in view of the donor shortage [1]. Accelerated fibrosis progression (FP) and recurrent cirrhosis (RC) have emerged as challenging complications after LT [2-8].

Today, in the new era of highly effective antiviral therapies, posttransplant recurrence of viral hepatitis C (HCV) has developed into a manageable condition, eliminating many cases of post-transplant cirrhosis. However, alcohol relapse, non-alcoholic steatohepatitis (NASH), autoimmune liver diseases and other underlying diseases can lead to rapid post-transplantation liver fibrosis and recurrent cirrhosis [3-6].

Several donor and recipient dependent clinical risk factors are known to be associated with FP, especially in patients with recurrent hepatitis C [9]. Immunosuppression and increasing donor age are held responsible for an accelerated rate of FP after LT [9-11]. These clinical risk factors can only in part explain why some patients develop very fast RC, within a few years after LT, while others show no significant fibrosis at the 5-year protocol biopsy [12]. Beside factors related to the
transplant procedure or the immunosuppressive management, genetic factors of the donor or recipient appear to influence FP after LT [13].

Few data regarding the effect of genetic predisposition for FP exist in post-transplant patients. Layden et al. [14] identified significant multivariate associations of FP in post-transplant HCV-infected patients with recipient gene variants of the interleukin 28B (IL28B), DEAD box protein 5 (DDX5), patatin-like phospholipase domain containing 3 (PNPLA3), suppressor of cytokine signaling-3 (SOCS3) and malectin (MLEC) genes [14].

A seven-gene signature, identified by genome wide association studies, is associated with FP after LT as reported by Huang et al. [15]. These genetic polymorphisms were used to establish a cirrhosis risk score (CRS). Patients with a CRS <0.5 were categorized as at low risk for FP; CRS values between 0.5 and 0.7 were associated with an intermediate risk and a CRS >0.7 was predictive of a high FP risk [15]. Importantly, several of the CRS genes have been confirmed functionally to be involved in hepatic fibrogenesis [16, 17].

Overall, 75% of the HCV-reinfected patients after LT displaying a high CRS >0.7 developed at least F2 fibrosis during follow-up, independently of known clinical risk factors such as donor age, sex of the recipient or acute rejection [18]. These data suggest that the genetic signature of the recipient predicts the likelihood of severe liver fibrosis development in the graft after HCV recurrence. However, the effect of the genetic risk profile of the donor organ on FP after LT as well the role of the recipient's CRS in patients with underlying liver diseases other than hepatitis C have not been investigated.

We therefore analyzed for the first time the relevance of the CRS for the development of fibrosis after LT in a large cohort of HCV-positive and HVC-negative LT recipients, including the impact of the donor genotypes.

**MATERIAL AND METHOD**

**Patients**

We evaluated 611 patients with LTs performed between 1998 and 2012 at the Interdisciplinary Transplant Center, University Medical Center Mainz. Inclusion criteria were: liver transplanted patients older than 18 years, Caucasians, complete histological data and genotyping available. Exclusion criteria were: retransplantation, successfully treated HCV infection after LT, fibrosing cholestatic hepatitis C, ischemic type biliary lesions, postoperative bile duct and vascular complications. Data was obtained from prospectively performed protocol liver biopsies and from prospectively maintained transplant databases. The selection process is shown in Fig. 1. Patients with HCV, hepatitis B viral (HBV) infection, hepatocellular carcinoma (HCC), occurred in the context of an underlying liver disease, alcoholic cirrhosis and other underlying diseases for LT (NASH, primary biliary cholangiopathy, primary sclerosing cholangitis, autoimmune hepatitis) were included.

All HCV-positive patients developed HCV-reinfection post-LT, diagnosed by positive HCV-RNA and characteristic laboratory findings. Alcoholic etiology before LT or alcohol relapse after LT were defined as alcohol intake >30 g/day for men and >20 g/day for women.

![Fig. 1. Selection process.](image)

Patients' data were provided by electronic medical records and patient charts. Medical history, data of physical examination and biochemical blood tests were obtained at least every 3–6 months within the first 5 years after LT, depending on the clinical status. Further data regarding donors' and recipients' demographics, patients' comorbidities, surgical complications and survival were recorded.

Finally, 491 patients met the inclusion criteria and were genotyped, after written informed consent. Our study followed the ethical guidelines of the Declaration of Helsinki and was approved by the local Ethics Committee of the State of Rhineland-Palatinate [no. 837.533.11(8075)].

**Histological Assessment**

Liver biopsies were performed in all donor livers before reperfusion and graft fibrosis >F1 before LT was excluded. Protocol liver biopsies were prospectively performed at 1 and 5 years after LT by Menghini needle, if patients gave their informed approval. In cases of abnormal laboratory values or clinical findings, an additional histological evaluation of the transplanted liver was performed. Liver specimens were assessed by two experienced pathologists. The second one was blinded to the results of the first pathologist and both were blinded to the patients' medical history. Staging of liver fibrosis was performed according to the scoring system of Desmet and Scheuer [19], using a scale from F0–F4. Significant fibrosis progression was assumed when a difference of at least one fibrosis stage to ≥F2 occurred between the biopsy at baseline (time of transplantation) and at follow-up. Cumulative incidences of fibrosis were defined as the probability to develop significant fibrosis (≥F2), proven by biopsy during the time of observation.
Immunosuppression

Patients received immunosuppression according to individual risk factors and comorbidities. The standard immunosuppressive regimen was a combination of a calcineurin inhibitor with mycophenolate mofetil. Target trough levels for tacrolimus were 5-7 ng/ml during the first 5 years and 3-5 ng/ml thereafter. For cyclosporine, the target trough level was 70-90 ng/ml during the first 5 years and 50-70 ng/ml thereafter. All patients received steroids in the first 3-6 months post LT.

Genotyping

Samples used for DNA analysis were collected prospectively. Genomic DNA from the recipient was extracted from 200 µl EDTA-blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Donor DNA was extracted from pre-implantation liver biopsies (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany). Determination of the genotypes of the following genes established in the CRS [15], was performed by real time PCR on a LightCycler® 2.0 device (Roche, Mannheim, Germany) using a commercial LightSNiP (SimpleProbe) assay purchased from Tib-MolBiol (Berlin, Germany), and according to the manufacturer’s recommendations: adaptor-related protein complex 3 S2 (AP3S2) (rs2290351), aquaporin 2 (AQP2) (rs2878771), antizyme inhibitor 1 (AZIN1) (rs62522600), degenerative spermatocyte homolog 1 (DEGS1-NVL) (rs4290029), syntaxin binding protein 5-like (STXBPSL5) (rs17740066), toll-like receptor 4 (TLR4) (rs4986791) and transient receptor potential cation channel M5 (TRPM5) (rs868277).

Samples were set up in a final volume of 10 µl, containing 1 µl of DNA solution (~50 ng), 1 µl of LightCycler FastStart DNA Master HybProbe Mix (Roche Molecular Biochemicals, Mannheim, Germany), 0.8 µl of MgCl2 (25 mM), and 0.5 µl of LightSNiP reagent mix (Tib-MolBiol, Berlin, Germany). Cycling conditions were as follows: initial denaturation (10 min 95°C) followed by 45 cycles of denaturation (10s at 95°C), annealing (10s at 60°C), and elongation (15s at 72°C). Subsequently, DNA melting was performed: 20s at 95°C, 20s at 40°C followed by 45 cycles of denaturation (10s at 60°C), and elongation (15s at 72°C). Thereafter, samples were cooled down to 40°C (ramp rate 20°C/s). When the probe melted off the template, fluorescence resonance energy transfer no longer took place and fluorescence was converted to melting peaks using software that plotted the negative derivative of fluorescence with respect to temperature.

CRS Algorithm

The CRS was calculated as described based on the SNPs of the 7 target genes (AP3S2, AQP2, AZIN1, DEGS1, STXBPSL5, TLR4 and TRPM5) using a naïve Bayes formula [15].

We stratified the patients into three CRS categories: <0.5, 0.5-0.7 and >0.7. To increase the statistical power, CRS-values <0.5 and 0.5-0.7 were pooled. Associations of CRS with clinical variables, histology and risk factors for fibrosis were analysed.

Statistical Analysis

All statistical analyses were performed using R version 3.4.2 [20]. Quantitative data are expressed as medians with interquartile ranges. Categorical variables are given as frequencies and percentages, respectively, and for the comparison of two or more groups Fisher’s exact test or a chi-square test was applied. Between-group differences for quantitative variables were assessed using the Wilcoxon rank sum test.

Cumulative incidences of fibrosis were calculated under consideration of death and retransplantation as competing risks using the cmprsk-package in R [21, 22]. Comparisons of cumulative incidence functions between subgroups were performed with special log-rank-tests that consider competing risks, again using the cmprsk-package [22]. Univariable and multivariable Cox proportional hazards models were used to evaluate the association of potential risk factors with the fibrosis risk in terms of cause-specific Hazard ratios (HR) for fibrosis. Thereby, the proportional hazards assumption was evaluated using Schoenfeld residuals.

All tests were performed two-sided. Our complete data analysis is exploratory. Hence no adjustments for multiple testing were performed. For all tests we used a 0.05-level to define statistically relevant deviations from the respective null hypotheses. However, due to the large number of tests, p-values should be interpreted with caution and in connection with effect estimates.

RESULTS

From all the 491 patients, patients were chosen according to genotype availability. The recipient's genotype was available in 442 patients (R-CRS group). In 201 patients, donor genotypes were available (D-CRS group). The two patient groups overlapped in 152 patients, in which both recipients' and donors' genotypes were known (Fig. 1). Demographic characteristics are presented in Table I for the two groups, defined according to the available genotype. Mean follow-up was 5.5 (3.2-9.4) years for the R-CRS group and 5.8 (2.7-8.3) years for the D-CRS group. Overall, 62% (n=274) of the recipients had a CRS>0.7 [25.6% (n=113) with a CRS <0.5, 36.4% (n=161) between 0.5 and 0.7], and 38% (n=168) of patients had a CRS score >0.7. Among patients with known donor genotype, 60.2% (n=121) of the patients had a CRS score ≤0.7 [23.8% (n=48) <0.5, 36.4% (n=73) between 0.5-0.7] and 39.8% (n=80) had a CRS >0.7. For HCV positive patients in the R-CRS group, the median CRS was 0.59 (interquartile range 0.41-0.75) and for HCV-negative recipients in this group it was 0.61 (0.51-0.77), p=0.156; for HCV positive patients in the D-CRS group it was 0.63 (0.52-0.77) and for HCV negative patients in the same group 0.61 (0.46-0.77), p=0.295.

Cumulative incidences of fibrosis and CRS

Development of fibrosis ≥F2 was documented in 117 (26.5%) patients from the R-CRS group and in 47 (23.4%) patients from the D-CRS group during follow-up. The prevalence of fibrosis ≥F2 differed according to the underlying liver disease. For example in the R-CRS group transplanted for HCC, we found significantly fewer patients with fibrosis ≥F2 (32/167) compared to patients transplanted for other underlying liver diseases (85/275), p=0.006. Cumulative incidences for fibrosis ≥F2 were higher in recipients with a D-CRS >0.7 in the whole
Table I. Patients' demographics

|                          | R-CRS† (n=442) | D-CRS‡ (n=201) |
|--------------------------|----------------|----------------|
| **Gender**               |                |                |
| Male (n; %)              | 300 (67.8)     | 141 (70.1)     |
| Female (n; %)            | 142 (32.2)     | 60 (29.9)      |
| **Age (years)**          |                |                |
| Median (quartiles)       | 55.1 (49.0-61.7)| 56.3 (50.2-63.9)|
| **BMI** (kg/m²)          |                |                |
| Median (quartiles)       | 24.3 (16.2-43.5)| 23.8 (15.3-44.8)|
| **HCV** (n; %)           |                |                |
| negative                 | 333 (75.3)     | 144 (71.6)     |
| Male                     | 225 (67.5)     | 100 (69.4)     |
| Female                   | 108 (32.4)     | 44 (30.5)      |
| Age (years; median, quartiles) | 55.8 (48.6-61.6) | 57.9 (51.5-64.1) |
| CRS <0.5; 0.5-0.7; >0.7  | 77 (23.1); 125 (37.5); 131 (39.3) | 38 (26.4); 52 (36.1); 54 (37.5) |
| positive                 | 109 (24.6)     | 57 (28.3)      |
| Male                     | 65 (59.6)      | 41 (71.9)      |
| Female                   | 44 (40.4)      | 16 (28.1)      |
| Age (years; median, quartiles) | 53.7 (49.1-62.3) | 53.1 (49.3-59.8) |
| CRS <0.5; 0.5-0.7; >0.7  | 36 (33.0); 36 (33.1); 37 (33.9) | 10 (17.5); 21 (36.9); 26 (45.6) |
| **HBV** (n; %)           |                |                |
| negative                 | 376 (85.1)     | 167 (83.1)     |
| positive                 | 66 (14.9)      | 34 (16.9)      |
| **HCC** (n; %)           |                |                |
| negative                 | 275 (62.2)     | 109 (54.2)     |
| positive                 | 167 (37.8)     | 92 (45.8)      |
| **Alcohol (n; %)**       |                |                |
| no                       | 241 (54.5)     | 119 (59.2)     |
| yes                      | 201 (45.5)     | 82 (40.8)      |
| **Others (n; %)**        |                |                |
| total                    | 66 (14.9)      | 28 (13.9)      |
| NASH§ (n; %)             | 29 (43.9)      | 14 (50)        |
| PBC§ (n; %)              | 16 (24.2)      | 1 (3.5)        |
| PSC§ (n; %)              | 15 (22.7)      | 5 (17.9)       |
| Autoimmune (n; %)        | 6 (9.0)        | 8 (28.6)       |
| **CRS (median; quartiles)** | 0.61 (0.48-0.77) | 0.62 (0.50-0.77) |
| <0.5                     | 113 (25.6)     | 48 (23.8)      |
| 0.5-0.7                  | 161 (36.4)     | 73 (36.4)      |
| >0.7                     | 168 (38.0)     | 80 (39.8)      |
| **Donor characteristics**|                |                |
| Donor age (years; median, quartiles) | 50.1 (39-62) | 50.9 (40-65) |
| Donor sex (males/females) | 224/218 | 95/106 | 57/52 | 26/31 | 167/166 |

CRS: cirrhosis risk score; †R-CRS: LT patients with available recipients' genotypes; ‡D-CRS: LT patients with available donors' genotypes; †R-M - body mass index; †HCV - hepatitis C virus; †HBV - hepatitis B virus; †HCC - hepatocellular carcinoma; §NASH - non-alcoholic steatohepatitis; ††PBC - primary biliary cholangiopathy; †††PSC - primary sclerosing cholangitis
The influence of different potential risk factors for fibrosis was analyzed by univariable Cox proportional hazard models (Table II). As expected, the strongest predictive factor for the development of fibrosis $\geq$F2 was HCV-infection ($p<0.001$). Further influencing factors were HBV infection in the R-CRS group, HCC in the HCV-negative R-CRS subgroup and recipient’s age in the HCV positive R-CRS and D-CRS subgroups. Donor’s CRS >0.7 was associated with higher hazard ratios (HRs) for fibrosis $\geq$F2 ($p=0.01$) in the whole D-CRS group and in HCV-negative patients ($p=0.03$).

Table II. Predictive factors for fibrosis $\geq$F2 (univariable analysis by the Cox-PH-model)

| Predictive factor         | HR1 (95% CI) | p2      |
|---------------------------|--------------|---------|
| R-CRS                     | n=442, n fib=117 |
| Sex male (ref. female)    | 0.96 (0.65-1.41) | 0.855  |
| Recipient age             | 0.98 (0.97-1.01) | 0.123  |
| Donor age                 | 1.00 (0.99, 1.02) | 0.113  |
| HBV positive (ref. negative) | 0.34 (0.17-0.70) | 0.003  |
| HCV positive (ref. negative) | 3.31 (2.29-4.76) | <0.001 |
| HCC positive (ref. negative) | 0.77 (0.53-1.14) | 0.204  |
| Alcohol yes (ref. no)     | 0.76 (0.51-1.11) | 0.163  |
| R-CRS-groups $>$0.7 (ref. $\leq$0.7) | 0.98 (0.67-1.43) | 0.938  |
| R-CRS, HCV neg n=333, n fib=55 |
| Sex male (ref. female)    | 0.67 (0.40, 1.11) | 0.120  |
| Recipient age             | 0.99 (0.97, 1.01) | 0.345  |
| Donor age                 | 1.01 (0.99, 1.02) | 0.226  |
| HBV positive (ref. negative) | 0.49 (0.28, 1.09) | 0.083  |
| HCC positive (ref. negative) | 0.54 (0.30, 0.99) | 0.046  |
| Alcohol yes (ref. no)     | 0.90 (0.55, 1.549) | 0.545  |
| R-CRS-groups $>$0.7 (ref. $\leq$0.7) | 0.93 (0.56, 1.57) | 0.812  |
| R-CRS, HCV pos n=109; n fib=62 |
| Sex male (ref. female)    | 1.65 (0.90, 3.03) | 0.104  |
| Recipient age             | 0.96 (0.92, 0.99) | 0.008  |
| Donor age                 | 1.01 (0.99, 1.03) | 0.341  |
| HBV positive (ref. negative) | 0.24 (0.03, 1.71) | 0.153  |
| HCC positive (ref. negative) | 0.75 (0.44-1.28) | 0.295  |
| Alcohol yes (ref. no)     | 1.26 (0.66, 2.38) | 0.482  |
| R-CRS-groups $>$0.7 (ref. $\leq$0.7) | 1.27 (0.73, 2.20) | 0.398  |
| D-CRS                     | n=201, n fib=47  |
| Sex male (ref. female)    | 0.81 (0.44, 1.49) | 0.517  |
| Recipient age             | 0.99 (0.97, 1.01) | 0.567  |
| Donor age                 | 1.01 (0.99, 1.03) | 1.170  |
| HBV positive (ref. negative) | 0.54 (0.21, 1.38) | 0.203  |
| HCV positive (ref. negative) | 3.37 (1.90, 6.00) | <0.001 |
| HCC positive (ref. negative) | 1.10 (0.63, 1.95) | 0.735  |
| Alcohol yes (ref. no)     | 0.77 (0.42, 1.43) | 0.424  |
| D-CRS-groups $>$0.7 (ref. $\leq$0.7) | 2.04 (1.15, 3.63) | 0.014  |
| D-CRS, HCV neg n=144, n fib=22  |
| Sex male (ref. female)    | 0.58 (0.24, 1.9)  | 0.208  |
| Recipient age             | 1.02 (0.99, 1.07) | 0.960  |
| Donor age                 | 1.01 (0.98, 1.03) | 0.258  |
To evaluate confounder-adjusted effect estimates for CRS, we performed further analyses using multivariable Cox proportional hazard models (Table III). For analysis of the HCV-negative D-CRS subgroup, we decided to include stepwise only two potential confounders in the multivariable analysis, due to the limited number of cases with relevant fibrosis in this subgroup (n=22). When evaluating this specific subgroup with different models including two further factors besides CRS, we observed in all combinations (7 models tested) that a D-CRS >0.7 was strongly associated with fibrosis ≥F2 compared to genotype scores ≤0.7 (p<0.05).

Early fibrosis and CRS

To investigate the association between CRS and early FP (≥F2), the available 1-year protocol biopsies after LT were analyzed. Interestingly, a donor’s CRS >0.7 was more frequently encountered in patients with ≥F2 in the 1-year protocol biopsy after LT, than in patients with F0 and F1. This was observed for the whole D-CRS group (p<0.001), as well as for the HCV-negative subgroup (p<0.001), while in the HCV-positive subgroup no effect of the donors’ genotype was evidenced (p=0.40), (Fig. 3A, B, C).

Among the patients in whom both the recipient’s and donor’s CRS were available, 94 patients agreed to protocol biopsy after one year. Fibrosis ≥F2 was encountered more frequently in patients with a D-CRS >0.7, in combination with any R-CRS (8/60) (p=0.034).

Severe fibrosis and CRS

With a limited number of recurrent cirrhosis, a R-CRS >0.7 was not predictive for the development of advanced fibrosis (≥F3) at 1 and 5 years after LT, neither in the whole, nor in the HCV-positive or HCV-negative R-CRS subgroup. However, a D-CRS >0.7 in HCV-negative patients showed a tendency towards a more frequent association with F3/F4 than a CRS value ≤0.7 (p=0.06) (Table IV, Supplementary material).

Individual SNPs and fibrosis ≥F2

The influence of individual SNPs on the development of fibrosis ≥F2 was analyzed by the Cox-proportional hazard

| Table II (continued) |
|-----------------------|
| HBV positive (ref. negative) | 0.86 (0.29, 2.54) | 0.788 |
| HCC positive (ref. negative) | 1.58 (0.68, 3.67) | 0.280 |
| Alcohol yes (ref. no) | 1.17 (0.50, 2.71) | 0.703 |
| D-CRS-groups >0.7 (ref. ≤0.7) | 2.59 (1.12, 6.01) | 0.025 |
| D-CRS, HCV pos | n=57, n fib =25 |
| Sex male (ref. female) | 1.19 (0.49, 2.85) | 0.69 |
| Recipient age | 0.94 (0.90, 0.99) | 0.015 |
| Donor age | 1.01 (0.98, 1.04) | 0.251 |
| HBV positive (ref. negative) | 0.42 (0.05, 3.10) | 0.394 |
| HCC positive (ref. negative) | 0.49 (0.21, 1.07) | 0.072 |
| Alcohol yes (ref. no) | 1.08 (0.37, 3.16) | 0.880 |
| D-CRS-groups >0.7 (ref. ≤0.7) | 1.17 (0.53, 2.56) | 0.699 |

1 HR – hazard ratio; 2 p – p value; 3 R-CRS: LT patients with available recipients’ genotypes; 4 n fib – number of fibrosis; 5 HBV – hepatitis B virus; 6 HCV – hepatitis C virus; 7 HCC – hepatocellular carcinoma; 8 D-CRS: LT patients with available donors’ genotypes.

| Table III. Predictive factors for fibrosis ≥F2 (multivariable analysis by the Cox-PH-model for CRS and potential confounders) |
|---------------------------------------------------------------|
| Group|Variables| HR (95% CI)| p|
| R-CRS (n=442, n fib =117)|Sex male (ref. female)| 1.11 (0.72 – 1.67) | 0.595 |
||Recipient age| 0.97 (0.95 – 0.99) | 0.033 |
||Donor age| 1.01 (0.99 – 1.02) | 0.053 |
||HBV positive (ref. negative)| 0.42 (0.19 – 0.89) | 0.023 |
||HCV positive (ref. negative)| 3.35 (2.25 – 5.01) | <0.001 |
||HCC positive (ref. negative)| 0.74 (0.49 – 1.13) | 0.169 |
||Alcohol yes (ref. no)| 0.89 (0.58 – 1.38) | 0.627 |
||R-CRS-groups >0.7 (vs. ≤0.7) | 1.09 (0.75 – 1.60) | 0.629 |
| D-CRS (n=201, n fib =47)|Sex male (ref. female)| 0.72 (0.36 – 1.38) | 0.330 |
||Recipient age| 0.98 (0.95 – 1.02) | 0.389 |
||Donor age| 1.01 (0.99 – 1.03) | 0.126 |
||HBV positive (ref. negative)| 0.78 (0.29 – 2.05) | 0.612 |
||HCV positive (ref. negative)| 3.30 (1.71 – 6.35) | <0.001 |
||HCC positive (ref. negative)| 0.97 (0.51 – 1.83) | 0.937 |
||Alcohol yes (ref. no)| 1.19 (0.58 – 2.43) | 0.630 |
||D-CRS-groups >0.7 (vs. ≤0.7) | 1.69 (0.93 – 3.06) | 0.079 |
| D-CRS, HCV negative (n=144, n fib =22)|Sex male (ref. female)| 0.97 (0.58 – 1.68) | 0.851 |
||Recipient age| 0.98 (0.95 – 1.02) | 0.389 |
||Donor age| 1.01 (0.99 – 1.03) | 0.389 |
||HBV positive (ref. negative)| 0.78 (0.29 – 2.05) | 0.612 |
||HCV positive (ref. negative)| 3.30 (1.71 – 6.35) | <0.001 |
||HCC positive (ref. negative)| 0.97 (0.51 – 1.83) | 0.937 |
||Alcohol yes (ref. no)| 1.19 (0.58 – 2.43) | 0.630 |
||D-CRS-groups >0.7 (vs. ≤0.7) | 1.69 (0.93 – 3.06) | 0.079 |

1 HR – hazard ratio; 2 p – p value; 3 R-CRS: LT patients with available recipients’ genotypes; 4 n fib – number of fibrosis; 5 HBV – hepatitis B virus; 6 HCV – hepatitis C virus; 7 HCC – hepatocellular carcinoma; 8 D-CRS: LT patients with available donors’ genotypes.

J Gastrointestin Liver Dis, March 2019 Vol. 28 No 1: 53-61
model, in an explorative manner. No individual SNP showed a HR significantly different from 1, neither in the R-CRS nor in the D-CRS group. However, the donor’s AZIN1 genotype (A/G vs. G/G) was associated with a relevant risk for fibrosis ≥F2 in the group of HCV-positive recipients (HR 4.01; 95% CI: 1.97-8.16; p=0.001). Furthermore, the donor’s STXBP5L genotype carried a higher risk for fibrosis ≥F2 in HCV-negative patients (HR = 2.72; 95% CI = 1.22-6.14; p= 0.02). A similar effect was shown for the TRPM5 genotype in HCV-positive patients of the D-CRS subgroup (HR=0.52; 95% CI=0.29-0.93; p=0.03).

**DISCUSSION**

Recurrence of fibrosis is an important cause of morbidity and mortality in LT-recipients [9]. Clinical risk factors for FP have been previously identified [12]. In the past few years several studies about the association of genetic factors with progression of fibrosis have been published [23-26].

Three retrospective longitudinal cohort studies evaluated the CRS as a predictive factor for FP in HCV positive patients [18, 27, 28]. Marcolongo et al. [27] analyzed a cohort of 271 Caucasian patients with mild chronic hepatitis C who underwent a diagnostic biopsy, and received a follow-up biopsy 5 years later. Only patients with F0-F2 at first biopsy were included and none of these patients received antiviral therapy during the study interval. Mean CRS was higher in patients with FP than in those without. Later, Trepo et al. [28] confirmed these results in a smaller, retrospective analysis of 56 Caucasian patients with chronic hepatitis C before LT, with F0-F1 at the first biopsy, and follow up after 5 years. The median CRS was higher in patients with fibrosis progression ≥F2. In the logistic regression model, only a CRS >0.7 had a significant influence on FP [28]. Do O et al. [18] assessed the predictive value of CRS after LT and evaluated 137 patients transplanted for HCV-induced cirrhosis, with the exclusion of HBV-coinfection or alcohol abuse. A CRS >0.7 was predictive for the development of F2 or F3 fibrosis in the protocol biopsies after one, three and five years. Moreover, the interval up to the development of fibrosis ≥F3 was shorter with a CRS >0.7 [18].

Our study went beyond these reports. First, it is the largest analysis on the prognostic relevance of CRS after LT to date. Second, in view of HCV-infection per se is a strong and independent predictor of fibrosis (with a HR > 3 in our patients), we also included HCV-negative patients in this analysis. Third, in all previous studies, the CRS was only determined in the transplant recipients, while the donor liver CRS likely has a prominent association with the risk of posttransplant FP. There is no previous report of the impact of D-CRS on severity of fibrosis after LT and only few donor genotypes are known to affect FP after LT [29]. We therefore determined for the first time the D-CRS to correlate it with graft fibrosis development.

When analysing the effect of the D-CRS according to HCV status, we found a significant association between ≥F2 and the donor’s CRS >0.7 in HCV-negative patients (p=0.03). In addition, cumulative incidences for fibrosis ≥F2 were higher in recipients with a donor CRS>0.7 in the whole D-CRS group (p=0.03) and by trend in the corresponding HCV-negative subgroup (p=0.06). The 1-year protocol biopsy showed a significant association of early fibrosis ≥F2 with a D-CRS >0.7 in all patients of the D-CRS group (p<0.001) and in the HCV-negative D-CRS patients (p<0.001). In contrast to do O et al.

**Fig. 3.** Fibrosis (≥F2 compared to <F2) in the protocol biopsy one year after LT according to the D-CRS. A: all D-CRS patients, n=121 protocol biopsies at 1 year, n=92 with <F2, 29 with ≥F2. B: HCV-negative patients of the D-CRS group, n=85 protocol biopsies at 1 year, n=69 with <F2, 16 with ≥F2. C: HCV-positive patients of the D-CRS group, n=36 protocol biopsies at 1 year, n=23 with <F2, n=13 with ≥F2. Patients receiving a donor liver with a CRS >0.7 showed a higher risk for significant early fibrosis ≥F2 than with a CRS ≤0.7 in the whole group and in the HCV-negative subgroup. D-CRS – patients in whom the donor genotype was available; HCV – hepatitis C virus; LT – liver transplantation.
[18], we did not find an association of the R-CRS scores >0.7 with higher cumulative incidences for fibrosis ≥F2 in biopsies.

There are some differences between our study and the previous publications in patients' selection, endpoint definition and data analysis [15, 18, 27, 28]. Huang et al. [15] performed a cross-sectional study that did not stratify patients according to the duration of HCV-infection and that did not address current dynamics of FP. The probability for development of severe fibrosis stage ≥F3 depends on the duration of infection and prior "second hits" other than the genetic risk that may not be active at the time of analysis [30]. Patients with F1 and F2 fibrosis were excluded and the prevalence of fibrosis ≥F3 was 90.9% in the validation cohort and 62% in the training cohort [15].

The other three studies had a longitudinal retrospective design and allow a better comparison to our study. However, the endpoints differed in all cohorts. The study by do O et al. [18] shows the strongest similarity to our study. Although patient groups seemed comparable with respect to recipient's age, men were more prevalent in their than in our HCV-positive R-CRS subgroup (63.5% vs. 59.6%), while our donors were older (median 50.4 years in our cohort vs. 42 years). Severe fibrosis (≥ F3) one year after LT was lower in our patients with 6.3% (28/442) in the total R-CRS group and 10% (11/109) in the HCV-positive R-CRS subgroup vs. 18.3% (21/115) in the HCV-positive patients reported by do O et al. [18]. High CRS >0.7 scores were more frequent in their than in our cohort (46.7 % in the HCV positive cohort of do O et al. vs. 38% in our whole R-CRS group and 34% in our HCV positive R-CRS subgroup). These differences between the two studies regarding the cohorts of HCV-positive LT recipients might explain why do O et al. [18] found an association of the recipient's CRS with fibrosis after LT, whereas this could not have been confirmed by us.

A limitation of our analysis using the CRS is that only some SNPs of the donor could be relevant for FP after LT, whereas others could influence fibrosis when belonging to the recipient. In the current era of safe and effective direct-acting antivirals, there is no relevant role for CRS in HCV-positive patients. However, the current functional studies related to the CRS-SNPs suggest fibrogenic potential independent of the cause of the liver disease [16, 17] and we found an effect of the donor's genotype mainly in HCV-negative patients.

Since fibrosis is the result of a complex interplay between donor and recipient features, considering recipient or donor variables alone might lead to an incomplete analysis, which is the major limitation of our study. The two only in part overlapping groups in our study could have been built up retrospectively only by random, to the extent to which genotyping was only by random, to the extent to which genotyping was available in this big real life cohort. Furthermore, we cannot provide longitudinal data for serological or ultrasound-based surrogate markers for fibrosis, since these methods were not extensively available at the beginning of the study. In our statistical analyses, the limited number of events with respect to the number of covariates may lead to some degree of overfitting. On the other hand, to prevent biased estimates, presumably important covariates should not be omitted in multivariable survival models.

CONCLUSIONS

Our data provide first evidence that the CRS genotype of the donor organ is associated with early fibrosis progression after LT, especially in HCV-negative patients. Further validation of the CRS and related scores based on donor and recipient genetic factors in well-defined prospective cohorts are warranted to assess FP and individual risk for recurrent cirrhosis and to improve therapeutic options for patients after LT.

Conflicts of interest: There is no conflict of interest. No funding sources were involved in the study design, in the collection, analysis and interpretation of data, in the writing or the decision to submit the article for publication.

Author contributions: A.Z., T.Z., F.D., D.S. designed the study, collected and analysed data, and wrote the paper. F.D., D.G., J.M., J.V. collected and analysed data, made critical revision. T.Z., D.S., P.R.G., H.L. analysed and interpreted data, and made critical revision. M.H.L., G.T. collected and analysed data, performed statistical analysis, drafting. F.A. and A.L. collected and analysed data, performed DNA extraction and genotyping, critical revision. A.S. performed histological evaluation of fibrosis, acquisition of data, critical revision. All authors approved the final version.

Acknowledgements: This work was supported by an intramural grant of the University of Mainz to T.Z. (Innenuniversitäre Forschungsförderung) and by an ERC Advanced Grant (Fibroimaging) to D.S.

We thank Larissa Herbel, 1st Department of Medicine and Ulis Suessdorf, Department of General, Visceral and Transplantation Surgery, University of Mainz for excellent technical assistance with the patients' samples.

Supplementary material: To access the supplementary material visit the online version of the J Gastrointestin Liver Dis at http://dx.doi.org/10.15403/jgld.2014.1121.281.crr

REFERENCES

1. Adam R, Karam V, Delvart V, et al. Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). J Hepatol 2012;57:675-688. doi:10.1016/j. jhep.2012.04.015
2. Levy G, Villamil FG, Nevens F, et al. REFINE: a randomized trial comprising cyclosporine A and tacrolimus on fibrosis after liver transplantation for hepatitis C. Am J Transplant 2014;14:635-646. doi:10.1111/ajt.12620
3. Rice JP, Eckhoff J, Agni R, Ghufran A, Brahmbhatt R, Lucey MR. Abusive drinking after liver transplantation is associated with allograft loss and advanced fibrosis. Liver Transpl 2013;19:1377-1386. doi:10.1002/lt.23762
4. Fosby B, Karlsen TH, Melum E. Recurrence and rejection in liver transplantation for primary sclerosing cholangitis. World J Gastroenterol 2012;18:1-15. doi:10.3748/wjg.v18.i1.1
5. Hytoroglou P, Gutierrez GA, Freni M, et al. Recurrence of primary biliary cirrhosis and development of autoimmune hepatitis after liver
17. Guo J, Loke J, Zheng F, et al. Functional linkage of cirrhosis-predictive single nucleotide polymorphisms of Toll-like receptor 4 to hepatic stellate cell responses. Hepatology 2009;49:960-968. doi:10.1002/hep.22697
18. do O NT, Eurich D, Schmitz P, et al. A 7-gene signature of the recipient predicts the progression of fibrosis after liver transplantation for hepatitis C virus infection. Liver Transpl 2012;18:298-304. doi:10.1002/lt.22475
19. Desmet VJ, Gerber M, Hoofnagle JH, Mans M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513-1520. doi:10.1002/hep.1840190629
20. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing 2017, Vienna, Austria. Available from: https://www.R-project.org/
21. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Statist Med 2007;26:2389-2430.
22. Bob Gray. cmprsk: Subdistribution Analysis of Competing Risks. R package version 2.2-7, 2014. Available from: https://CRAN.R-project.org/package=cmprsk
23. Eurich D, Bahro M, Boas-Knoop S, et al. Transforming growth factor beta1 polymorphisms and progression of graft fibrosis after liver transplantation for hepatitis C virus--induced liver disease. Liver Transpl 2011;17:279-288. doi:10.1002/lt.22190
24. Eurich D, Boas-Knoop S, Struecker B, Neuhaus R, Neuhaus P, Bahra M. Genetic variants of STAT-4 affect the development of graft fibrosis after liver transplantation for HCV-induced liver disease. Transplantation 2013;95:203-208. doi:10.1097/TP.0b013e318277e2f6
25. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401. doi:10.1038/nature08309
26. Zimmermann T, Hoppe-Lotichius M, Körner A, et al. The recipient CYP2D6 allele 4-associated poor metabolizer status correlates with an early fibrosis development after liver transplantation. Transpl Int 2011;24:1059-1067. doi:10.1111/j.1399-6584.2011.01305.x
27. Marcolongo M, Young B, Dal Pero F, et al. A seven-gene signature (cirrhosis risk score) predicts liver fibrosis progression in patients with initially mild chronic hepatitis C. Hepatology 2009;50:1038-1044. doi:10.1002/hep.23111
28. Trepo E, Potthoff A, Pradat P, et al. Role of a cirrhosis risk score for the early prediction of fibrosis progression in hepatitis C patients with minimal liver disease. J Hepatol 2011;55:38-44. doi:10.1016/j.jhep.2010.10.018
29. Dunn W, O’Neil M, Zhao J, et al. Donor PNPLA3 rs738409 genotype affects fibrosis progression in liver transplantation for hepatitis C. Hepatology 2014;59:453-460. doi:10.1002/hep.26758
30. Berenguer M, Schuppan D. Progression of liver fibrosis in post-transplant hepatitis C: mechanisms, assessment and treatment. J Hepatol 2013;58:1028-1041. doi:10.1016/j.jhep.2012.01.014
Table IV (Supplementary data). Biopsy-data, at 1 and 5 years after the LT, according to the recipient’s (R-) and the donor’s (D-) CRS. Comparison between patients without fibrosis (F0) and those with severe fibrosis ≥F3 (p values were calculated with the Fisher’s exact test).

| Biopsy data at follow-up time points | 1 year | 5 years |
|-------------------------------------|--------|--------|
| Fibrosis stage (Desmet and Scheuer) | 0      | 3/4    | 0      | 3/4    |
| **R-CRS\(^1\), HCV\(^2\) positive** |        |        |        |        |
| CRS <0.5                           | 11     | 3      | 7      | 3      |
| 0.5-0.7                            | 12     | 2      | 4      | 3      |
| >0.7                               | 8      | 6      | 6      | 4      |
| p\(^3\) (CRS ≤0.7; CRS >0.7)       | 0.135  |        | 1.00   |        |
| **R-CRS, HCV negative**            |        |        |        |        |
| CRS <0.5                           | 23     | 4      | 21     | 10     |
| 0.5-0.7                            | 51     | 7      | 41     | 16     |
| >0.7                               | 49     | 6      | 31     | 23     |
| p (CRS ≤0.7; CRS >0.7)             | 0.796  |        | 0.145  |        |
| **R-CRS, total**                   |        |        |        |        |
| CRS <0.5                           | 34     | 7      | 28     | 13     |
| 0.5-0.7                            | 63     | 9      | 45     | 19     |
| >0.7                               | 57     | 12     | 37     | 27     |
| p (CRS ≤0.7; CRS >0.7)             | 0.672  |        | 0.136  |        |
| **D-CRS\(^4\), HCV positive**     |        |        |        |        |
| CRS <0.5                           | 3      | 0      | 0      | 0      |
| 0.5-0.7                            | 9      | 2      | 0      | 2      |
| >0.7                               | 12     | 2      | 0      | 0      |
| p (CRS ≤0.7; CRS >0.7)             | 1.00   |        | 0.99   |        |
| **D-CRS, HCV negative**            |        |        |        |        |
| CRS <0.5                           | 22     | 2      | 7      | 0      |
| 0.5-0.7                            | 30     | 1      | 7      | 0      |
| >0.7                               | 15     | 4      | 9      | 2      |
| p (CRS ≤0.7; CRS >0.7)             | 0.06   |        | 0.18   |        |
| **D-CRS, total**                   |        |        |        |        |
| CRS <0.5                           | 25     | 2      | 7      | 0      |
| 0.5-0.7                            | 39     | 3      | 7      | 2      |
| >0.7                               | 27     | 6      | 9      | 2      |
| p (CRS ≤0.7; CRS >0.7)             | 0.17   |        | 0.99   |        |

\(^1\) R-CRS: LT patients with available recipients’ genotypes; \(^2\) HCV – hepatitis C virus; \(^3\) p – p value; \(^4\) D-CRS: LT patients with available donors’ genotypes.