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HOST AND VIROLOGICAL FEATURES OF HCV INFECTION IN LIVER TRANSPLANT SETTING

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ABSTRACT

Cirrhosis due to hepatitis C (HCV) infection, with or without hepatocellular carcinoma (HCC), is the leading indication for liver transplantation (LT) worldwide. Recurrent HCV infection of the allograft is universal if the virus is detectable at the time of transplant, with variable clinical course. In one third of liver recipients recurrent HCV infection lead to cirrhosis in the graft within only 5 years after LT. A small proportion of recipients can develop cholestatic HCV (CHC), a severe form of recurrence carrying a poor prognosis. In the last years, the approval of interferon-free (IFN-free) regimens for the treatment of chronic HCV has been a major step forward in Transplant Hepatology. The safety and efficacy of direct acting antivirals (DAAs) allow to treat patients awaiting LT as well as individuals with HCV recurrence after LT. Here we present two studies including HCV-infected patients in the peri-transplant setting. In the first study we aimed to assess the persistence of HCV-RNA in liver explants of HCV-infected patients treated with an IFN-free regimen on the waitlist for LT, and to analyze if its presence was associated with relapse after LT. Moreover, we investigated the potential role of the innate immune response on the persistence of HCV-RNA in the liver. Residual HCV-RNA was detected in liver explant in 67% of HCV-infected patients treated with DAAs on the waitlist, and with serum HCV-RNA negative at transplant. Nevertheless, HCV-RNA persistence in liver explant does not seem to be associated with virological relapse after transplantation, except in cases where liver HCV-RNA concentrations are high. Interestingly, we also found that the intrahepatic IFN pathway is down-regulated in patients treated with DAAs.

In the second study we investigated virological mechanisms involved in the pathogenesis of cholestatic HCV (CHC) after LT, assessing the HCV quasispecies evolution and presence of specific mutations within the NS5B region, before and after LT, using ultra-deep pyrosequencing. Patients included in this study were at the extreme spectrum of hepatitis C recurrence: mild disease with an excellent long-term outcome and patients with CHC with a poor outcome. We showed that in patients with CHC, the predominant virus strain outgrow the

others and generates a relatively uniform quasispecies. In these patients, HCV appears to acquire an increased fitness in the new graft, since we found that the master sequence present before LT propagated in most of CHC patients after LT, but not in patients with mild HCV recurrence. We cannot exclude that specific mutations within HCV genome of patients with CHC may explain the high viral replication capacity and fitness. Indeed, we found specific mutations in the HCV genome of some patients with CHC, but our sample was not sized to show significant differences with individuals with mild hepatitis C recurrence. A further step forward could be to introduce these mutations in in vitro models to test their impact on virus replication/fitness.

In conclusion, the antiviral treatment of hepatitis C infection with DAAs before liver transplantation seems the ideal option in the liver transplant setting. However, in patients treated with DAAs on the waiting list, who have high explant HCV-RNA concentrations and a downregulation of IFN-pathway, another therapeutic strategy should be choose in order to avoid HCV recurrence after LT.

In patients who develop a cholestatic hepatitis C recurrence after LT, the application of specific mutations of HCV polymerase gene in *in vitro* models might be crucial for a better understanding of the mechanisms underlying this severe form of HCV recurrence.

RIASSUNTO

La cirrosi epatica dovuta all'infezione dell'epatite C (HCV), associata o meno ad epatocarcinoma, è l'indicazione principale al trapianto di fegato. La ricorrenza dell'infezione nel fegato trapiantato è universale nei pazienti HCV positivi al momento del trapianto. Un terzo dei riceventi presenta un quadro cirrosi epatica nei 5 anni successivi al trapianto di fegato e una parte minore può sviluppare una forma severa di ricorrenza, associata ad una prognosi infausta, chiamata epatite colestasica fibrosante (CHC). Negli ultimi anni, l'utilizzo delle terapie antivirali senza interferone per il trattamento dell'epatite C ha segnato un traguardo importante dell'epatologia dei trapianti. Infatti l'efficacia e la sicurezza dei farmaci antivirali ad azione diretta (DAAs) permette di trattare i pazienti con cirrosi HCV-correlata in lista d'attesa per trapianto e i pazienti trapiantati che hanno sviluppato una ricorrenza dell'infezione dopo trapianto. In questa tesi presentiamo due studi che includono pazienti affetti da epatite HCV nell'ambito del trapianto di fegato. Nel primo studio lo scopo è stato quello di determinare la persistenza del genoma virale (HCV-RNA) nel fegato espiantato di pazienti con cirrosi HCV-correlata, che avevano ricevuto un trattamento antivirale con DAAs, durante la lista d'attesa per trapianto di fegato. Abbiamo inoltre valutato il ruolo dell'immunità innata nella persistenza dell'HCV-RNA nel fegato espiantato dopo trattamento antivirale. Abbiamo dimostrato che, nonostante il trattamento, il genoma virale persisteva nel fegato espiantato della maggior parte dei pazienti. Tuttavia, la presenza di HCV-RNA non è risultata associata al fallimento del trattamento, ossia alla ricorrenza dell'infezione HCV dopo trapianto, tranne che nei pazienti in cui vi era una elevata quantità di genoma virale nel fegato espiantato. Interessante è stato vedere come in questi pazienti trattati con DAAs ci fosse una ridotta attivazione della via di segnalizzazione cellulare dell'interferone.

Nel secondo studio sono stati indagati specifici meccanismi virologici coinvolti nella patogenesi dell'epatite colestasica C (CHC), la forma più severa di ricorrenza dell'epatite C dopo trapianto di fegato. L'evoluzione delle popolazioni virali che circolano normalmente in un

unico individuo, chiamate quasispecie virali, e la presenza di specifiche mutazioni nella regione codificante per la polimerasi del virus (NS5B) sono state studiate utilizzando una tecnica molto sensibile e innovativa, quale il sequenziamento massivo di nuova generazione. Sono stati inclusi nello studio due gruppi di pazienti: pazienti che avevano sviluppato la CHC e pazienti che avevano presentato una forma lieve di ricorrenza dell'epatite C dopo trapianto, inclusi nel gruppo di controllo. Abbiamo visto che nei pazienti con CHC uno specifico ceppo virale dominava sugli altri, generando una quasispecie virale omogenea. In questi pazienti il virus aveva acquisito una maggiore "fitness", confermato anche dal fatto che la sequenza maggioritaria presente prima del trapianto si manteneva anche dopo trapianto. Invece, nei pazienti con una ricorrenza lieve questo non avveniva, e la quasispecie virale appariva più disomogenea dopo trapianto. Nei pazienti con CHC, la presenza di specifiche mutazioni del gene NS5B potrebbero spiegare il comportamento del virus, che replicando ad alti livelli induce un danno cellulare severo. Abbiamo identificato alcune mutazioni della regione NS5B nei pazienti con CHC ma, probabilmente per il piccolo campione incluso nello studio, tali mutazioni non erano significativamente più presenti nei pazienti con CHC rispetto al gruppo di controllo. L'obiettivo successivo sarà quello di utilizzare queste mutazioni per creare un modello *in vitro*, che permetta di confermare il loro impatto sulla replicazione del virus e la patogenesi della CHC.

In conclusione, in pazienti trattati con i nuovi potenti farmaci antivirali durante la lista d'attesa per trapianto, che presentano alti livelli di HCV-RNA nell'espianto e con una ridotta risposta immunitaria innata, dovrebbe essere adottata un'altra strategia terapeutica dopo trapianto.

Nei pazienti che sviluppano una epatite colestatica HCV correlata dopo trapianto di fegato, lo studio e l'utilizzo di mutazioni presenti nel genoma virale possono essere utili allo scopo di definire i meccanismi alla base di questa severa forma di ricorrenza dell'epatite C dopo trapianto di fegato.

LIST OF ABBREVIATIONS

DAA: direct acting antiviral

SOF: sofosbuvir

RBV: ribavirin

LDV: ledipasvir

DCV: daclatasvir

BMI: body mass index

W: week of antiviral treatment

TND: target not detected

HCV: hepatitis C virus

LT: liver transplantation

IFN: interferon

SVR: sustained virological response

pTVR: post-transplant virological response 12 weeks after liver transplantation

GT: HCV genotype

ISGs: interferon-stimulated genes

MxA: myxovirus resistance A (MxA) protein

OAS1: 2'–5' oligoadenylate synthetase-1 oligoadenylate synthetase-1

HVPG: hepatic-venous portal gradient

CHC: cholestatic hepatitis C

MELD: model of end stage liver disease

FDC: fixed-dose combination

LSM: liver stiffness measurement

GGT: gamma-glutamyl transferase

ALP: alkaline phosphatase

ALT: alanine aminotransferase

AST: aspartate aminotransferase

HCC: hepatocellular carcinoma

UDPS: ultra-deep pyrosequencing

PCR: polymerase chain reaction

1. GENERAL INTRODUCTION: HEPATITIS C INFECTION IN THE LIVER TRANSPLANT SETTING

Cirrhosis secondary to chronic hepatitis C (HCV), with or without hepatocellular carcinoma (HCC), is the leading indication for liver transplantation (LT) worldwide (1,2). Recurrent HCV infection of the allograft is universal if the virus is detectable at the moment of transplant surgery. One of the main characteristics of HCV recurrence after LT is the accelerated course of the disease when compared to immunocompetent patients. Approximately one third of liver recipients will progress to liver cirrhosis in the graft within only 5 years after LT. This accelerated fibrosis rate impacts both the allograft and recipient survival, which is significantly reduced when compared with non-HCV liver recipients, as reported by several large registry analyses (3). During transplantation, the HCV-infected liver is removed and replaced by a graft, so consequently, serum HCV RNA decreases dramatically, even disappears, in most patients after transplantation. Despite this initial decrease, there may be a rapid increase in HCV viral load within hours after graft reperfusion, and the serum HCV load may reach pre-transplantation levels in only a few days. Acute hepatitis (biochemical and histological) generally occurs in the first 3–4 months, before the establishment of chronic hepatitis and its sequelae (4,5). The clinical outcome of recurrent HCV infection after LT may be variable, and depends of (or is related to) several factors that take place in the first period after LT. The presence of significant fibrosis (fibrosis stage ≥ 2) or significant portal hypertension (HVPG≥ 6 mmHg) in the graft 1 year after LT identifies patients with severe HCV recurrence (6-10). These patients, defined as “rapid fibrosers”, have a higher risk of clinical decompensations than patients with lower stages of fibrosis and normal portal pressure at the same time point. A very severe form of HCV recurrence, fortunately uncommon, is called cholestatic HCV that is characterized by marked cholestasis with or without cirrhosis or rapidly expanding sinusoidal fibrosis, leading to high risk of liver failure and mortality (11-14). Early recognition of CHC is

crucial to its successful treatment. Very little is still known about the pathophysiology of cholestatic hepatitis C recurrence and the ways in which it differs from other more conventional forms of aggressive hepatitis C.

In the last years, the approval of interferon-free regimens for the treatment of chronic hepatitis C has been a major step forward in Hepatology (15-27). The safety and efficacy of the combination of several direct acting antivirals (DAAs), compared to interferon-containing regimens, has opened hope for groups of patients in whom interferon-based regimens were contraindicated or in whom these regimens had a very limited efficacy and poor tolerance: patients awaiting liver transplantation and individuals with hepatitis C recurrence after transplantation. The results from clinical trials and real-life cohort showed sustained virological response rates (SVR) up to 90% along with high safety profile in cirrhotic patients and in liver recipients (15-27). The aim of the treatment during the waiting list is to achieve HCV-RNA negative or SVR before LT to avoid HCV infection of the graft. On the other hand, the main goal of treating HCV infection in transplanted patients is to prevent liver injury related to HCV recurrence, finally improving both patient and graft survival when virus eradication is achieved (28).

Here we present 2 studies including patients in the 2 different scenario of HCV infection: before and after liver transplantation. In the first study we evaluated virological and host- related features in HCV-infected cirrhotic patients awaiting a transplant, undergoing an IFN-free regimens during the waiting list. In the second study we investigated virological mechanisms involved in the pathogenesis of cholestatic HCV after LT in HCV-infected recipients.

2. RESIDUAL HCV-RNA IN LIVER EXPLANTS OF PATIENTS AWAITING LIVER TRANSPLANTATION TREATED WITH AN INTERFERON-FREE REGIMEN

2.1. Background and aims

The introduction of DAAs in the hepatitis C armamentarium has completely changed the management of patients with chronic HCV infection, particularly in the peri-transplant setting. The main goal of treatment in patients awaiting a LT is to prevent HCV graft infection, by eradicating the virus in the serum and liver (28). A full treatment course is not always possible before transplantation, as waiting time is unpredictable. Fortunately, patients treated with the available IFN-free regimens have rapid declines in viral load, with most achieving undetectable serum HCV-RNA within 4 weeks of the initiation of therapy (18). However, the duration of treatment necessary to clear HCV from infected liver cells in patients with advanced cirrhosis using IFN-free regimens is unknown. Indeed, the only data assessing the persistence of HCV-RNA in liver explants of patients treated while on the waiting list are from the IFN era (29).

The main purpose of the present study was to assess the presence of HCV-RNA in liver explants of HCV-infected patients treated with an IFN-free regimen on the waitlist for LT, and to analyze if its presence was associated with relapse after LT. Secondarily, we investigated the potential role of the innate immune response on the persistence of HCV-RNA in the liver, by analyzing the intrahepatic expression of specific interferon-stimulated genes (ISGs).

2.2. Patients and methods

2.2.1. Patient population and collected data

We enrolled patients who had received an IFN-free regimen while on the waiting list for LT with an available explanted liver. Patients who achieved SVR before LT, those with detectable HCV-RNA in serum at time of transplantation, and those who died after LT without reaching enough follow-up to assess a SVR (see below) were excluded. The following clinical and virological variables were recorded: demographical, treatment regimen and duration, baseline

viral load and HCV-RNA concentrations during treatment and at time of LT, HCV genotype, recipient IL28B genotype, liver disease severity scores (MELD and Child-Pugh scores). The study was approved by the Ethical Committee of the Hospital Clínic of Barcelona and by the institutional review board or independent ethics committees at participating sites; patients gave written inform consent. The study was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements (for patients included in the 2 clinical trials).

2.2.2. Antiviral treatment regimens

Patients received different IFN-free regimens: 1) Patients included in a phase 2, open-label, pilot study received SOF administered orally once daily at a dose of 400 mg, along with RBV administered orally as a divided dose according to body weight. The treatment durations were 24 or 48 weeks (18); 2) patients included in a phase 3 clinical study received ledipasvir (LDV) and SOF administered orally once daily at a fixed-dose combination (FDC) of 90/400 mg, along with RBV administered orally as a divided dose according to body weight and liver disease stage for 12 or 24 weeks (27); 3) patients who were not part of a clinical trial underwent treatment with SOF and RBV for at least 24 weeks, SOF plus daclatasvir (DCV) 60 mg daily and RBV for 24 weeks, or SOF plus simeprevir (SMV) 150 mg daily and RBV, for 12 weeks. RBV was administered orally as a divided dose according to body weight and liver disease stage (starting at 600 mg if clinical decompensation was present).

2.2.3. Serum HCV-RNA quantification

Quantification of serum HCV-RNA was performed by COBAS® Ampliprep® HCV Test. Sustained virological response (SVR) after LT (pTVR12) was defined as HCV-RNA target not

detected (TND) (HCV-RNA < 15 IU/mL) that persisted 12 weeks after LT. The HCV genotype was determined by sequence analysis of the NS5B region of the HCV genome.

HCV-RNA extraction and quantification from liver explant tissue

Tissue samples from liver explants were collected at the time of LT and preserved in RNA-later solution (Qiagen, Hilden, Germany) at −80ºC until use. Total RNA was extracted from 20-30 mg of starting tissue using the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. After tissue disruption and homogenization, 18 µl of IC (Internal Control, Abbott RealTime HCV assay) were added to each sample. RNA was finally eluted in 88 µl of RNase-free water and stored at -80°C until the quantification. RNA concentration and quality were assessed with the NanoDrop 1000 spectrophotometer (ThermoScientific, NanoDrop products, Wilmington, DE) and the RNA was stored at -80o C. HCV-RNA was determined using the Real-Time HCV assay (Abbott Molecular, Des Plaines, IL) and the Abbott m2000rt instrument. Since this assay is intended for serum samples, we have adapted the protocol for HCV-RNA quantification for liver tissue samples. HCV-RNA concentration in liver was expressed as copies of HCV-RNA per µg of total RNA.

2.2.4. Assay calibration and limit of HCV-RNA detection

A calibration curve was generated by adding known serial amounts of in vitro transcribed HCV-RNA (from JFH1 isolate) to total RNA purified from HCV-negative liver explant samples (alcoholic cirrhosis, NASH and HBV-related cirrhosis). The HCV-RNA panel ranged from 1x106 to 1x101 copies per reaction and each panel sample was tested in duplicate (the average measured values were compared with the expected HCV-RNA levels). The concentration of HCV-RNA in a tissue sample was calculated from the stored calibration curve and expressed as HCV-RNA copies/µg of total RNA. In order to evaluate the sensitivity of the assay, serial samples containing 100 (n= 5), 20 (n= 6), 10 (n= 38), 5 (n= 26), 2.5 (n= 19), 1

(n= 30), 0.1 (n= 21) HCV-RNA copies were tested. Specificity was assessed by testing 56 liver samples obtained from 6 HCV-negative LT recipients (3 patients with alcoholic cirrhosis, 2 patients with HBV-related cirrhosis and 1 patient with NASH). The limit of detection was established in 6 copies per reaction.

2.2.5. Relative quantification of OAS1 and MXA genes expression

Relative quantification of the myxovirus resistance A (MxA) protein and 2'–5' oligoadenylate synthetase-1 oligoadenylate synthetase-1 (OAS-1) gene expression was performed using the Applied Biosystems® 7500 Real-Time PCR System and the 7500 software v.2.0.1 (Applied Biosystems, Carlsbad, CA), using high quality RNA extracted from liver explant samples as explained above. The FirstChoice® Human Liver Total RNA (Life Technologies, Ambion, Carlsbad, CA) was used as reference for target genes (OAS1 and Mx1). The human RPLP0 (Large Ribosomal Protein) gene expression was used as endogenous control. Single-stranded cDNA from 200 ng total RNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and random primers according to manufacturer´s instructions. The assay probes for OAS1 (Cat. # 4331182, FAM-MGB), Mx1 (Cat. # 4331182, FAM-MGB) and RPLP0 (Cat. # 4310879E, VIC® -TAMRA) were purchased from ThermoFischer Scientific (Carlsbad, CA). Prior to relative quantification of the OAS1 and Mx1 genes, an initial validation relative standard curve (CT at y-axis, linear scale vs. RNA quantity at x-axis, log scale) was performed to validate the PCR efficiencies of the OAS1, Mx1, and RPLP0 genes. Quantitative PCR reactions were performed using the SensiFAST™ Probe Lo-ROX Kit (Bioline, Singapore) according to manufacturer´s instructions. Parallel RNA standard curves were obtained for the 3 above mentioned genes. Therefore, the comparative CT method (the ΔΔCT method) was used to assess the relative expression of the OAS1 and Mx1 genes in each liver explant sample. Quantification of the target genes OAS1 and Mx1 genes were performed in triplicate. Final data are expressed as mean values of 3

measurements (±SD) and represent the relative (fold) gene expression of the OAS1 and Mx1 genes in each liver explant sample in comparison to the reference RNA mentioned above and are corrected to the endogenous control gene expression.

The expression of the OAS1 and Mx1 genes was also measured in 2 control groups:1) patients with detectable HCV-RNA in serum at time of LT (n=13) (8 non treated patients and 5 individuals undergoing an IFN-free regimen), 2) patients with undetectable HCV-RNA in serum who were receiving an IFN-based regimen at time of transplantation (n=6).

2.2.6. Statistical analysis

Continuous variables are depicted using median and interquartile ranges (IQR), and categorical variables are expressed as absolute numbers and percentages. Univariate analysis was performed to define the differences in clinical and virological features between positive and negative explant livers and, among positive, between responders and relapsers. For categorical variables, differences between groups were assessed using Fisher's exact test, while differences among quantitative variables were analyzed with Mann-Whitney test. Probit analysis has been used to determine the HCV-RNA limit of detection of the Real-time PCR. All differences and associations were considered significant at a 2-sided p-value of <0.05. Statistical analyses were performed with SPSS, version 18 (SPSS, Chicago, IL).

2.3. Results

Liver explants were available from 48 patients awaiting a liver transplant who received at least 1 dose of a specific IFN-free regimen: 38 patients received SOF and RBV as part of a phase II clinical trial (18), 6 patients underwent treatment with SOF/LDV with RBV as part of a phase III clinical trial (27) and 4 patients received SOF with RBV (n=1), SOF plus DCV and RBV (n=2) or SOF plus SMV and RBV (n=1), as part of a real-life cohort at the Liver Unit, Hospital Clinic (Table 1). Nine of these 48 patients were excluded from the study for the following

reasons: death before the post-LT 12-week time point (n=3), SVR12 achieved before LT (n=2), detectable serum HCV-RNA at the moment of the transplant (n=4). Thus, 39 liver explants were considered for the final analysis, as illustrated in Figure 1.

The clinical and virological characteristics of patients are summarized in Table 1. Seventy-two percent of patients were male, 77% were infected by HCV genotype 1, and nearly 80% had non-CC IL28B polymorphisms. Twenty-four of the 39 (62%) patients with evaluable liver explants had compensated liver cirrhosis (Child-Pugh class A). The median MELD score was 9. The median treatment duration at the time of hepatectomy for LT was 17 weeks and 70% of patients had serum undetectable HCV RNA by the third week of treatment.

2.3.1. Persistence of HCV-RNA in liver explant

HCV-RNA was quantified in liver explant samples, as described in Patients and Methods. HCV-RNA was detected and quantified in 26 of the 39 liver explants (67%) whereas it was undetectable in the remaining 13 (33%). Comparing patients with an HCV-RNA positive liver explant and patients with HCV-RNA negative liver explant, the first group received a shorter treatment course (14 vs 21 weeks, respectively, p=0.014) and remained HCV-RNA undetectable in serum before transplant for a shorter period of time (61 vs 99 days, respectively, p=0.013) (Table 1). Regarding viral kinetics, time to reach HCV-RNA target not detected (TND), as well as HCV-RNA viral decay slopes (from baseline to weeks 1, 2, and 3) were similar in the 2 groups.

Thirty-three out of 39 patients (85%) achieved pTVR and 6 patients (15%) presented recurrent HCV infection after LT. HCV-RNA was detected in liver explants from 22 (67%) of the 33 responders and in 4 (67%) of the 6 relapsers. Thus, we did not find any association between the persistence of HCV-RNA in liver explants and the post-transplant HCV recurrence (Figure 1).

Considering only those 26 patients with residual HCV-RNA in the liver explant, the intrahepatic HCV-RNA concentration was significantly higher in those who relapsed after LT (n=4) compared to patients who achieved pTVR (n=22), (23 vs 3 copies/µg total RNA, respectively, p=0.016) (Table 2). The amount of residual HCV-RNA in the liver explant was not significantly different in patients with Child-Pugh ≥B7 and MELD ≥12 compared to those with CTP <B7 and MELD <12, respectively (p=0.108 and p=0.164, respectively). Explant HCV-RNA concentrations did not differ between individuals treated with SOF and RBV and those few patients who received more than one DAA.

2.3.2. Intrahepatic expression of MxA and OAS-1

As expected, among patients treated with an IFN-containing regimen (n=6), the expression of ISGs was significantly higher than in the other groups (Figure 2a and 2b). Similarly, patients with positive serum HCV-RNA at time of transplant (n=13, including non-treated patients and patients undergoing an IFN-free regimen) had a significantly higher expression of both MxA and OAS1 compared to patients with undetectable serum HCV-RNA at time of transplantation (n=39) (Figure 2a and 2b). Among patients with positive serum HCV-RNA at time of transplantation, we did not find a significant correlation between the viral load and the levels of MxA and OAS1 within the liver (r=0.5, p=0.09 and r=0.49, p=0.1, respectively).

Interestingly, among our study cohort (patients with undetectable serum HCV-RNA at time of LT), the intrahepatic innate immune response, as measured by the abundance of MxA and OAS-1 RNA, was similar in individuals with (n=24) and without (n=13) HCV-RNA in the liver explants (p=0.626 and p=441, respectively) (Figure 3a and 3b).

2.3.3. Analysis of NS5B mutants according to the presence of HCV-RNA in liver explants

In a subgroup of 30 patients who were part of the phase II clinical trial (18) we assessed the presence of emergent amino acid substitutions within the NS5B region, in serum, at baseline and at time of relapse, by population sequencing and deep sequencing (18). Essentially, we were interested to explore if the distribution of mutants within this region was similar or not between patients with and without HCV-RNA in the explant (Figure 4). Resistance to sofosbuvir is conferred by the S282T substitution in NS5B (30). S282T was not detected in any of the patients at baseline or virologic failure. L159F and V321A have been identified as treatment emergent variants associated with sofosbuvir treatment (31,32). Interestingly, 3 out of 6 relapsers had L159F detected as major species at baseline and relapse, whereas this variant was not present in any of the responder patients. RBV associated variants T390I, F415Y were present at baseline in 3 patients with detectable HCV-RNA in liver explants but were not detectable at relapse time point (Figure 4). In the 2 relapsers with a negative HCV-RNA explant these polymorphisms were present as double or triple mutants (Figure 4).

3. VIROLOGICAL FEATURES IMPLICATED IN THE DEVELOPMENT OF CHOLESTATIC HEPATITIS C AFTER LIVER TRANSPLANTATION

3.1. Background and aims

An uncommon and severe variant of hepatitis C recurrence after liver transplantation (LT) is a cholestatic graft injury termed cholestatic hepatitis C (CHC), usually present between 3 to 6 months after LT (11-14). CHC, whose frequency ranges between 2% and 14%, is typically characterized by marked cholestasis and rapid progression to graft failure. Patients who develop CHC have among the highest viral loads in serum and liver, with levels of viral replication significantly higher than in the non-immunosuppressed state. The ILTS Consensus Conference in 2002 defined CHC using several features that should be present to make the diagnosis (11). Variables that may influence the progression of disease include, but are not limited to, HCV-RNA level at the time of LT, amount of administered immune suppression, type of immunosuppression, early histological changes of the allograft and donor age. In the IFN-era, many patients with CHC were too sick to be considered eligible for antiviral treatment based on Peg-IFN and RBV, or they had to stop therapy earlier due to side effects. Fortunately, the use of new DAAs, particularly in IFN-free regimens, has drastically changed the poor prognosis of this severe form of hepatitis C recurrence (33). Results from several clinical studies, using different combinations of DAAs in CHC patients, reported curative response rate up to 100% (17). In these patients severe alteration of liver tests (bilirubin, albumin, INR) returned to normal a few weeks after treatment initiation and viral clearance. Despite the fact that most DAAs did not show clinically significant interactions with immunosuppressive drugs (Cyclosporine and Tacrolimus), an antiviral treatment within the first 6 months after LT might be difficult to manage. Indeed, early after LT, patients are still under strong immunosuppression, at risk of opportunistic infections, not uncommonly recovering or being treated from surgical complications and undergoing treatment with multiple drugs.

There is still a limited understanding of the mechanisms leading to the development of CHC (34). Hepatocellular injury in CHC is believed to be a cytopathic effect of HCV which, replicating at high level, directly induces cellular degeneration in a short period of time, causing progressive and rapid liver failure. Since HCV is a highly replicative virus, in each replication cycle, every possible point mutation and many double mutations are generated and may be present within the virus population at any time. HCV exists as a heterogeneous population of viral quasispecies, defined as a number of closely related yet unique genomic RNA viral sequences produced over time in an individual (35,36). Sequencing of multiple clones of HCV cDNA has shown that the composition of the HCV quasispecies changes after LT. The role of complexity and diversity of viral quasispecies in the pathogenesis of HCV recurrence after LT has been investigated in previous studies, showing controversial results (37-40). The contradictory results in the literature could be explained by the use of different techniques assessing viral quasispecies, variability in those methods evaluating quasispecies complexity, diversity, and divergence, and to the heterogeneity of patients' characteristics. Of the above mentioned limitations, it is important to state that the methods used to study HCV quasispecies in LT setting were suboptimal, limiting the possibility to describe low frequency viral populations and to detect mutations potentially implicated in the development of CHC recurrence. Therefore, the analysis of HCV quasispecies diversity and complexity with a sophisticated methodology can be relevant to better understand the pathogenesis of CHC. We hypothesized that the genetic evolution of the HCV region encoding for the NS5B polymerase, and the selection/fixation of specific mutations in the HCV non-structural regions might be implicated in the pathogenesis of CHC, explaining the high virus replication in CHC patients.

Therefore, the aim of this study was to analyze in patients with CHC, compared to a control group HCV quasispecies evolution and presence of specific mutations within the NS5B region, before and after LT, using ultra-deep pyrosequencing.

3.2. Patients and methods

3.2.1. Patient population and collected data

All patients with end-stage liver disease or hepatocellular carcinoma (HCC) secondary to chronic hepatitis C infection who underwent LT at Hospital Clinic of Barcelona between 05/1999 and 03/2012 were considered for this study. Patients were followed by a standard protocol and relevant variables were collected prospectively and included in a database, after approval by the Ethical Committee of the Hospital Clínic of Barcelona. All patients signed an inform consent to this purpose. Patients with the diagnosis of CHC were included in CHC group and patients with mild HCV recurrence were included in the control group. The diagnosis of CHC was performed according to previous published criteria (11): bilirubin >6 mg/dL, GGT and ALP ≥ 5 ULN, very high serum HCV-RNA, and typical histology of CHC in the absence of biliary/arterial complications. Mild HCV recurrence was defined by absent or minimal fibrosis (F0-F1) or liver stiffness measurement (LSM) below 8.7 kPa, during a follow-up of 5 years after LT. No patient received antiviral therapy during the study period.

Regarding recipient and donor variables, we considered: recipient age and gender, donor age, episodes of acute graft rejection (including episodes requiring prednisone-based treatment), biochemical liver tests (bilirubin, GGT and ALP) within 3 months after LT, immunosuppression (tacrolimus or cyclosporine), recipient IL28 polymorphisms, HCV genotype and viral load before and after LT (1-3 months after LT).

Two serum samples for each patient were used (a total of 44 samples): 1 sample taken at the moment of transplant and 1 sample obtained between months 1 and 3 after LT (in patients with CHC, at the moment of the highest viral load).

3.2.2. HCV-NS5B fragment amplification for UDPS

HCV RNA was extracted from 140 µL of serum by manual RNA extraction using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany), as specified by the manufacturer. The measures

to prevent contamination suggested by Kwok and Higuchi {Kwok, 1989 683 /id} were strictly applied. RT-PCR was performed using Transcriptor One Step RT-PCR Kit (Roche Applied Science Basel, Switzerland), using 20pmol of both the sense primer 5Bo8254 (5'-CNTAYGAYACCMGNTGYTTTGACTC-3') and the antisense primer 5Bo8707 (5'-TTNGADGAGCADGATGTWATBAGCTC-3'). Reverse transcription was performed at 50ºC for 30 min followed by a 2-step PCR reaction including: denaturing for 7 min at 94ºC; a first step of 10 cycles of 10 s at 94ºC, 30 s at 50ºC, and 1 min at 68ºC; a second step of 25 cycles of 10 s at 94ºC, 30s at 50ºC and 1 min at 68ºC, increasing extension time 5 s per cycle; a final 7-min step at 68ºC. Hemi-Nested-PCR was performed using FastStart High Fidelity PCR System, dNTPack (Roche Applied Science Basel, Switzerland). Briefly, 5µL from the previous PCR were amplified by a second PCR using a pair of primers which were composed by universal M13 forward (M13f) and M13 reverse (M13r) at 5' ends followed by a specific fragment (italic face) complementary of the HCV PCR product amplified in the first round. Nested-PCR conditions were the following: denaturing for 2 min at 95ºC; 35 cycles of 30 s at 95ºC, 30s at 55ºC and 1 min at 72ºC; a final 7-min step at 72ºC.

For sample identification, the final product of the hemi-nested amplification, was subjected to 15-cycles of re-amplification using primers composed by a complementary universal M13 primer (either sense or antisense) followed by a Roche's Validated Multiplex Identifier (MID) and with oligoA or B at 5' or 3' end of the sense or antisense primer, respectively.

Amplification products were analyzed by 1.8% agarose gel electrophoresis and negative controls (amplifications in the absence of RNA) were included in parallel to ensure the absence of contamination by template nucleic acids. PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA), quantified using the PicoGreen assay (Invitrogen, Carlsbad, CA, USA), and analyzed for quality using the BioAnalyzer DNA 1000 LabChip (Agilent, Santa Clara, CA, USA) prior to UDPS.

3.2.3. Ultra-deep pyrosequencing and data treatment

Massive sequencing was performed in the GS-Junior 454/Roche platform (Roche, Branford, CT 06405, USA), using titanium chemistry, which enables sequencing of 400- to 500-nt fragments (GS Junior Titanium Sequencing Kit), following manufacturer recommendations.

The data used for the analysis was the FASTA files obtained from the 454 GS Junior system's software, which applies stringent quality controls on each sequenced nucleotide to guarantee the integrity of the full length of the amplicon. Briefly, the sequences were first demultiplexed by identifying MID and specific primer for each strand, and quality filtered by excluding all haplotypes with more than two Ns (any base), three gaps, not covering the full amplicon, or with an an identity below 67% relative to the master sequence (defined here as the most abundant haplotype in the corresponding population). The accepted haplotypes with Ns and/or gaps were repaired by comparison with the dominant haplotype.

3.2.4. HCV quasispecies analysis

The quasispecies complexity of each sample was computed based on the haplotypes common to the forward and reverse strands and above 1% of abundance after the quality filter. Exclusion of haplotypes present at frequencies below 1%, eliminates from the analysis both putative artefactual sequences and haplotypes with the lowest relative fitness; the procedure renders the most frequent sequences at each time point for comparison purposes. The computed complexity indices were the nucleotide diversity defined as the average number of differences between all possible haplotype pairs, corrected by their frequencies in the population and the mutation frequency defined as the sum of the differences between each haplotype and the dominant haplotype (corrected by frequency).

In order to investigate the presence of specific amino acid mutations in viral population that propagated after LT, all HCV-NS5B sequences (before and after LT) from both groups of patients were aligned using ClustalX.

3.2.5. Statistical analysis

Continuous variables are depicted using median and interquartile ranges (IQR), and categorical variables are expressed as absolute numbers and percentages. Univariate analysis was performed to define the differences in clinical and virological features between patients with CHC and patients with mild HCV recurrence after LT (control group). For categorical variables, differences between groups were assessed using Fisher's exact test, while differences among quantitative variables were analyzed with Mann-Whitney test. All differences and associations were considered significant at a 2-sided p-value of <0.05. Statistical analyses were performed with SPSS, version 18 (SPSS, Chicago, IL).

3.3. Results

3.3.1. Patients characteristics

During the study period, 515 adult patients with end stage liver disease related to HCV infection underwent liver transplantation. During the first year after LT, 200 patients (53%) were classified as severe hepatitis C recurrence, either by the presence of significant fibrosis or portal hypertension during the first 12 months following LT or by the occurrence of a cholestatic hepatitis C or severe acute hepatitis. The remaining 173 (46%) patients were classified mild hepatitis C recurrence. Twenty-two patients were selected for the study inclusion: 13 patients with a diagnosis of CHC and 9 patients with mild HCV recurrence (control group). Clinical and virological characteristics of patients are summarized in Table 3.

3.3.2. Quasispecies analysis

At time of LT, the quasispecies diversity and the mutation frequency were similar between patients with CHC and patients with mild hepatitis C recurrence (Fig 5a and 5b).

After LT, the genetic diversity decreased in both groups of patients, but this decrease was more pronounced in the patients with cholestatic hepatitis, showing a marked homogenization of the quasispecies (Figure 6). These results were confirmed by the mutation frequency, that significantly decreased after LT only in the CHC patients. This result suggests the selection of variants more similar to the master sequence, and probably more fitted (Figure 7).

Regarding the propagation of HCV quasispecies after transplantation, interestingly, we found that in the majority of patients with CHC (69%) the master sequence present at the moment of LT propagated efficiently and remained as the dominant sequence after LT. This behavior was seen in only 1 patient included in the control group (11%) ($p=0.026$) (Figure 8a and 8b).

We also investigated the presence of amino acid mutations analyzing 24 different polymorphic sites in the NS5B region. We identified 9 amino acid mutations present in both groups in a not significantly different proportion, and 4 amino acid mutations (N215S, T267F, C316N, A246T) present only in CHC patients (Figure 9).

4. DISCUSSION

Patients with HCV-related cirrhosis awaiting a LT were one of the most difficult-to-treat populations in the era of IFN-based antiviral treatment. This scenario has dramatically changed in the last year, as patients on the waitlist for LT are treated with more efficacious and safe IFN-free regimens (15-17). In the first study including patients who received IFN-free therapy during the waiting list for LT (18), the number of consecutive days with HCV RNA target not detected (TND) prior to transplant has been identified as highly predictive of post-transplant virological response (pTVR), According to these study, patients awaiting LT should wait at least 30 days after serum HCV-RNA clearance to prevent HCV graft infection. This rule, however, is not applicable in the real-life as the waiting time is unpredictable. So the question is if all HCV patients on the waiting list should receive antiviral treatment, because even though the main source of HCV infection (the liver) is removed at transplant, the persistence of residual HCV-RNA in liver explants might impact SVR after LT in patients undergoing treatment while on the waitlist. Since there are no studies assessing the time required to clear HCV from liver cells in patients undergoing IFN-free therapy, we aimed to assess the presence of HCV-RNA in liver explants of HCV-infected patients treated with an IFN-free regimen on the waitlist for LT. In our study, unexpectedly, two thirds (67%) of patients had residual HCV-RNA in their liver explants. It is true that patients with HCV-RNA positive explants received a shorter treatment course and remained serum HCV-RNA undetectable before transplant for a shorter period of time than patients with HCV-RNA negative liver explants. Nevertheless, there was a substantial proportion of individuals with residual HCV-RNA in their explants who had cleared HCV from serum by week 3 of therapy and remained so for an additional number of weeks (median 10) before transplantation. This is a novel and unexpected finding, especially considering that the persistence of HCV-RNA in liver explants did not prevent the achievement of a SVR after LT in the majority of patients.

We found that the presence of HCV-RNA in liver explants did not impact on virological response after LT, however, among patients with residual HCV-RNA in the liver, there was a higher amount of tissue HCV-RNA in those who relapsed after LT compared to patients who responded. Despite the small number of patients, the data suggest that the intrahepatic levels of HCV-RNA could be predictive of posttransplant HCV recurrence among patients who are serum HCV-RNA negative at the time of transplantation. A potential explanation is that in those patients with higher HCV-RNA concentrations in the liver, tiny and intermittent amounts of virions (below the sensitivity of the quantification assays) are released into the serum and cause a virological relapse after LT. From a pathogenic point of view, we cannot exclude that a subpopulation of virus can persist despite of antiviral therapy. Indeed, the sequestration of HCV genome in membranous webs may be a potential mechanism by which the virus avoids degradation and clearance from hepatocytes (41). Some data support the idea that this sort of compartmentalization protects the viral RNA from host defense mechanisms, leading to a small fraction of HCV RNA to persist in these structures despite both antiviral therapy and host immune responses. The integrity of HCV genome present in liver explants could not be studied due to its low concentration and thus, we cannot exclude that due to the mechanism of action of sofosbuvir (chain terminator), the residual HCV-RNA found in the liver was composed of incomplete HCV genome fragments.

Our findings are in contrast with those reported for a cohort of HCV-infected patients awaiting LT undergoing Peg-IFN/RBV therapy who had undetectable HCV-RNA at time of transplantation, which demonstrated a correlation between the presence of residual HCV-RNA in the explant and HCV recurrence after LT (29). HCV-RNA was detected in the liver explants of 4 (80%) out of 5 relapsers, but only in 2 (17%) of 12 patients who achieved pTVR. The interferon system is a crucial component of the innate immune response against HCV and exogenous IFN could enhance the clearance of infected liver cells. IFN activity is mediated by the induction of intracellular proteins, such as the MxA protein and OAS-1. As expected, we

found that the intrahepatic expression of MxA and OAS-1 was significantly higher in patients treated with IFN (though being serum HCV-RNA negative), compared to patients with undetectable serum HCV-RNA undergoing an IFN-free regimen. The stronger exogenous induction of the intrahepatic IFN-α pathway in patients undergoing IFN therapy explains this finding. Intrahepatic expression of MxA and OAS1 was higher in individuals with detectable HCV-RNA in serum (reflecting active liver HCV replication) compared to patients with undetectable serum HCV-RNA who were undergoing an IFN-free treatment. Moreover, in the latter group, the intrahepatic down-regulation of the IFN pathway was seen both in patients with and without residual HCV-RNA in liver explants. Our results are in agreement with those recently published by Meissner et al (42), who demonstrated, for the first time, that HCV clearance achieved during IFN-free treatment with SOF/RBV is accompanied by hepatic down-regulation of type II and III IFNs, their receptors, and ISGs. Down-regulation of ISGs was associated with on-treatment viral suppression and occurred regardless of treatment outcome, since all patients achieved virologic suppression on therapy. However, patients who achieved SVR had higher intrahepatic expression of ISGs at end-of-treatment compared with patients who relapsed. The authors suggested that patients able to reestablish IFN homeostasis by the end-of-treatment may be more likely to achieve an SVR, whereas patients who fail to restore homeostasis may be more prone to viral relapse. We observed a lower amount of MxA RNA in livers from patients who relapsed after transplantation, but the difference did not reach statistical significance (data not shown).

In conclusion, in this study we demonstrated that, despite the use of effective antiviral treatments, residual HCV-RNA remains present in liver explants of a significant proportion of HCV-infected patients treated on the waitlist, also considering that the intrahepatic IFN pathway is down-regulated in these patients. Nevertheless, HCV-RNA persistence in liver explant does not seem to be associated with virological relapse after transplantation, except in cases where liver HCV-RNA concentrations are high.

A proportion of patients with decompensated cirrhosis awaiting a LT cannot receive a full antiviral treatment before transplant (18). In some of them is even not possible to start antiviral therapy due to their severe clinical status. These patients should wait to be treated after LT (26). Indeed, several studies have shown excellent safety and efficacy results of DAAs-based treatment in HCV-infected liver transplant recipients (17).

HCV infection is accelerated after LT, leading to worse graft and patient survival in patients undergoing LT for HCV-related cirrhosis than in those transplanted for other causes (3,28). Disease progression is highly variable and while some patients develop cirrhosis within a few years, the disease remains mild and does not progress in others. The mechanisms that determine these outcomes are not well known, although a complex interplay between host and viral factors is probably involved. Cholestatic hepatitis C constitutes the more severe form of HCV recurrence with almost universal mortality (13). It is typically characterized by early HCV recurrence with marked cholestasis, rapid fibrosis progression leading to graft failure within the first 1 to 2 years after transplantation. Because of its relatively low frequency, only scarce clinical data are available, and risk factors for CHC development remain unresolved (33). Older donors, corticosteroid treatment for acute cellular rejection, high levels of HCV RNA after LT, non-C/C recipient IL-28B genotype have all been implicated in previous reports (13). HCV quasispecies were also implicated in the pathogenesis of CHC, with contradictory results (37-40). We investigated for the first time, using a high sensitive method, HCV quasispecies evolution of HCV NS5B region, coding for the HCV polymerase, in patients with CHC compared to a control group. We showed that the complexity of HCV quasispecies in pretransplant serum did not correlate with the posttransplant disease course, as in both groups, CHC and control, the distribution of HCV quasispecies was similar. On contrary, after LT, patients with CHC presented a homogenization of HCV quasispecies of NS5B, more pronounced than patients with mild HCV recurrence (whose viral quasispecies had a greater post-LT quasispecies complexity). Messaguer et al. (40) previously demonstrated that there

was an inverse correlation between the diversification of the NS5B region and the severity of liver damage during the first year after LT. They showed that patients with mild recurrence had a more elevated genetic evolution of the NS5B region along with lower HCV-RNA concentrations, whereas a significant proportion of patients with severe histological recurrence presented high levels of viremia along with little genetic evolution of NS5B. Another study showed that the degree of diversification of another HCV genome region, hypervariable region-1 (HVR-1) of the HCV-E2 domain, was inversely correlated with the severity of HCV recurrence (39). Sequencing the same HCV genome region, Pessoa et al. (37) found no differences in terms of HCV complexity before LT between patients with or without CHC; however, quasispecies diversity (inter-sample diversity) was greater in transplanted patients over time, especially in those with severe recurrent disease. Using an innovative methodology, ultra-deep sequencing, we confirmed and expanded on the previous data.

In the context of many divergent genomes present in the infecting viral quasispecies, one or a few viruses may outgrow the others and generate a relatively uniform quasispecies. One can argue that the low grade genetic evolution of NS5B observed in CHC might be related to the weak immune pressure against HCV (34) resulting from the immunosuppressive therapy. However, we did not show any significant difference between the two groups in terms of immunosuppression. After LT, emerging HCV quasispecies requires adaptation to the new graft, and those propagated viral variants that most efficiently replicate become predominant (36). In addition, new mutants with increased fitness for the graft may emerge and compete with pre-LT variants. Supporting the latter, we found that the master sequence present before LT propagated in most of CHC patients after LT, but not in patients with mild HCV recurrence. Similarly, using a different method, (HTA and nucleotide sequencing) Sullivan et al. (38) demostrated that quasispecies major variants present in pretransplant serum were efficiently propagated after liver transplantation in 3 patients with severe HCV recurrence but not in the 2 patients with mild recurrence. It is possible that some circulating viruses in the initial

quasispecies after LT were defective and only a very small proportion of the viruses had the capability of initiating infection of the graft. Indeed, we identified specific mutations in patients with CHC, but our sample sized did not allow to prove a distinct pattern between both groups of patients. We cannot exclude that some specific strains have a higher pathogenic potential than others, due to the presence of the above-mentioned mutations Importantly, patients included in this study were at the extreme spectrum of hepatitis C recurrence: mild disease (individuals with an excellent long-term outcome) and patients with CHC. In the latter group, viral load was extremely high (and significantly different than in control patients). Thus, it appears that the virus infecting the graft from patients with CHC had a clear replication advantage. Nevertheless, to prove this hypothesis we would need to incorporate such mutations in subgenomic replicons or full-length HCV isolates and demonstrate a high replication capacity.

In conclusion, despite the high efficacy of DAAs-based antiviral treatment in HCV-infected patients awaiting LT, we showed persistence of HCV-RNA in the liver explant of a high proportion patients. This, however, does not seem to impact on posttransplant HCV recurrence, except for patients with high explant HCV-RNA concentrations. We showed a downregulation of IFN-signaling in patients treated with DAAs awaiting a LT, that could explain, at least in part, some cases of HCV recurrence.

In patients who develop a cholestatic hepatitis C (CHC) recurrence after LT the virus replicates at high level in the graft. We found that the master sequence present before LT propagated in most of CHC patients after LT, but not in patients with mild HCV recurrence. The latter supports a replication advantage in the viral quasispecies of patients with CHC. We identified some specific NS5B mutations in virus genome of patients with CHC, which could explain a greater pathogenic potential. In order to demonstrate this hypothesis, the next step should be to incorporate such mutations in a subgenomic replicons or a full-length HCV isolate.

5. TABLES

Variables ***n (%)*** ***Median (IQR)***	***Entire cohort (IFN-free regimen) n=39***	***Explant HCV-RNA undetectable n=13***	***Explant HCV-RNA detectable n=26***	***p****
DAA regimen				
SOF+RBV (7)	30	10	20	
SOF+LDV+RBV (8)	5	0	5	
SOF+RBV or SOF/DCV or SMV+RBV	4	3	1	
Gender (male)	28 (72%)	9 (69%)	19 (73%)	0.801
Age (years)	58 (55-62)	61 (55-63)	57 (55-59)	0.189
BMI	27 (24-31)	26 (22-31)	27 (25-32)	0.432
Treatment duration [weeks]	17 (13-24)	21 (16-26)	14 (10-22)	0.014
Time prior LT with serum RNA negative [days]	77 (46-122)	99 (76-171)	61 (15-118)	0.013
Viral load at baseline [Log IU/L]	6.1 (5.6-6.5)	6 (5.8-6.7)	6 (5.6-6.5)	0.988
Genotype G1	30 (77%)	8 (62%)	22 (85%)	0.107
GT1a	12 (31%)	4 (31%)	8 (31%)	
GT1b	18 (46%)	4 (31%)	14 (54%)	
GT2	4 (10%)	2 (15%)	2 (8%)	
GT3	4 (10%)	2 (15%)	2 (8%)	
GT4	1 (3%)	1 (8%)	0 (0%)	
IL28B CT/TT	27 (75%)	9 (82%)	18 (72%)	0.531
ALT at baseline (IU/L)	79 (45-96)	83 (68-106)	70 (43-92)	0.250
Child-Pugh before treatment				0.185
	24 (62%)	9 (69%)	15 (58%)	
Class A	10 (26%)	3 (23%)	7 (27%)	
ClassB	5 (13%)	1 (8%)	4 (15%)	
Class C				
MELD before treatment	9 (7-11)	9 (7-10)	10 (8-11)	0.143
HCV-RNA TND at W2	7 (20%)	3 (30%)	4 (16%)	0.350
HCV-RNA TND at W3	21 (70%)	7 (70%)	14 (70%)	1.00
HCV-RNA TND at W4	24 (68%)	6 (60%)	18 (72%)	0.490
MxA (fold-change)	1.8 (1.3-3.5)	2.06 (1.3-3)	1.73 (1.5-4)	0.611
OAS (fold-change)	10.1 (7.5-14)	9.07 (7-12)	10.1 (8-15)	0.436

Table 1. Clinical and virological characteristics of patients treated with an IFN-free regimen (n=39) on the waiting liver for liver transplantation (n=39), comparing those with and those without detectable HCV-RNA in liver explant, by univariate analysis. Categorical variables are shown as n (%) and quantitative variables as median (IQR).

Variables n=26	pTVR12 n=22	Relapse n=4	p
Gender (male)	17 (77%)	2 (50%)	0.258
Age (years)	57 (55-59)	57 (53-62)	0.948
BMI	27 (24-31)	27 (25-31)	0.882
Treatment duration [weeks]	15 (11-23)	10 (4-22)	0.481
Time prior LT with serum RNA negative [days]	64 (34-121)	14 (13-74)	0.201
Viral load at baseline [Log IU/L]	6 (5.6-6.4)	6 (5.3-6.7)	0.887
Copies HCV-RNA/reaction	3 (1.7-5.9)	23 (8.6-69)	0.016
Genotype G1	18 (82%)	4 (100%)	0.354
IL28B CT/TT	14 (67%)	4 (100%)	0.174
ALT at baseline (IU/L)	72 (54-92)	43 (36-97)	0.266
MELD at baseline	11 (8-13)	8 (7-10)	0.201
Child-Pugh	6 (5-8)	6 (5-7)	0.423
HCV-RNA TND at W2	19 (90%)	2 (50%)	0.106
HCV-RNA TND at W3	10 (62%)	4 (100%)	0.143
HCV-RNA TND at W4	14 (67%)	4 (100%)	0.174
OAS	10 (7-18)	12 (10-15)	0.510
MxA	1.8 (1.5-4.4)	1.7 (1.3-4.6)	0.642

Table 2. Clinical and virological characteristics the 26 patients with an HCV-RNA positive liver explant comparing those who achieved pTVR and those who relapsed, by univariate analysis. Categorical variables are shown as n (%) and quantitative variables as median (IQR).

Variables N (%), median (IQR)	CHC n=13	Mild recurrence (controls) n=9	p
Recipient gender (male)	9 (69%)	7 (78%)	0.627
Recipient age (years)	61 (51-66)	50 (47-56)	0.056
Donor age (years)	62 (45-65)	35 (28-49)	0.003
HCV genotype (G1)	11 (84%)	7 (77%)	0.368
Recipient IL28B (CC)	1 (7%)	3 (33%)	0.091
Immunosuppression (TAC)	10 (76%)	4 (44%)	0.199
Acute rejection	0 (0%)	3 (33%)	0.050
Viral load before LT (log IU/L)	5.6 (5-6)	5.5 (5-5.7)	0.720
Viral load after LT (log IU/L)	8 (7.7-8)	5.6 (5.6-6.4)	0.002
GGT (IU/L)	1513 (765-2708)	216 (23-520)	<0.001
AP (IU/L)	860 (553-1141)	255 (153-358)	<0.001
Bilirubin (ug/mL)	6.4 (3.5-16)	1.1 (0.6-1.4)	0.001

Table 3. Clinical and virological characteristics of patients, comparing patients with cholestatic HCV (CHC) and patients with mild HCV recurrence (controls).

6. FIGURES

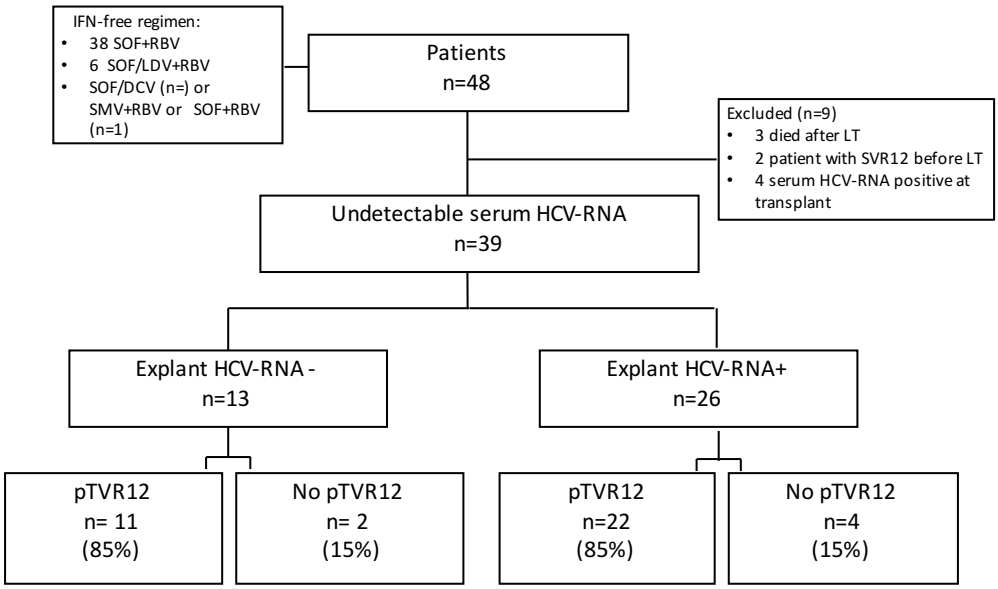

Figure 1. Flow chart of patients included in the study

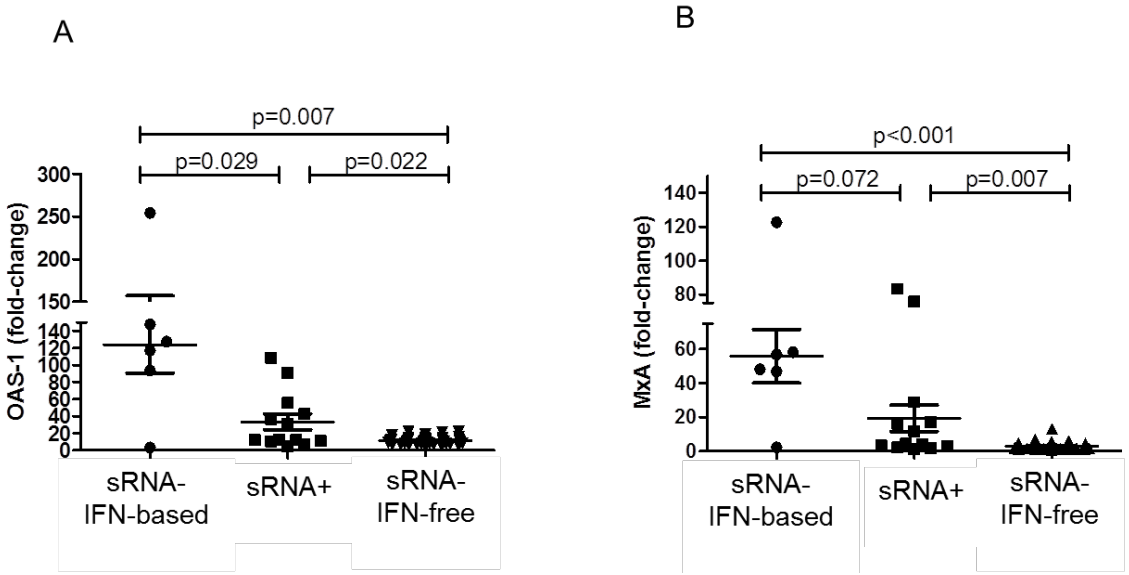

Figure 2. Expression of OAS1 (A) and MxA (B) RNA in liver explants of different groups of patients: individuals undergoing interferon-based therapy and serum HCV-RNA undetectable (sRNA- IFN-based), patients with positive HCV-RNA in serum (sRNA+) and patients undergoing an IFN-free regimen and undetectable HCV-RNA in serum (sRNA IFN-free). The amount of OAS1 and MxA RNA are depicted in the y axis as fold-change in comparison to the reference RNA and corrected to the endogenous control gene expression.

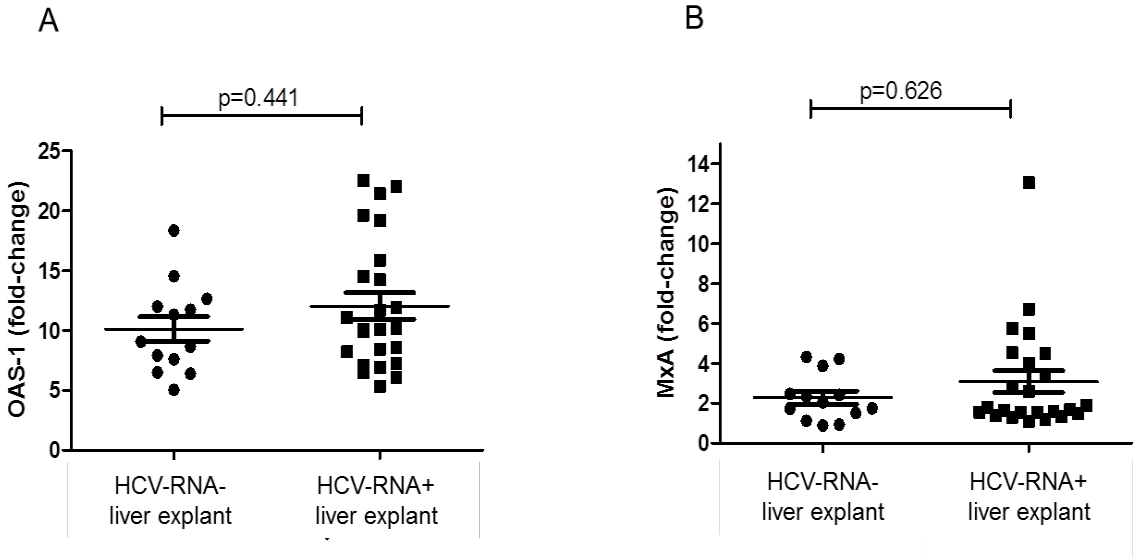

Figure 3. Expression of OAS1 (A) and MxA (B) RNA in liver explants of patients undergoing IFN-free therapy and undetectable serum HCV-RNA, comparing those with an HCV-RNA positive and with a negative liver explant. The amount of OAS1 and MxA RNA are depicted in the y axis as fold-change in comparison to the reference RNA and corrected to the endogenous control gene expression.

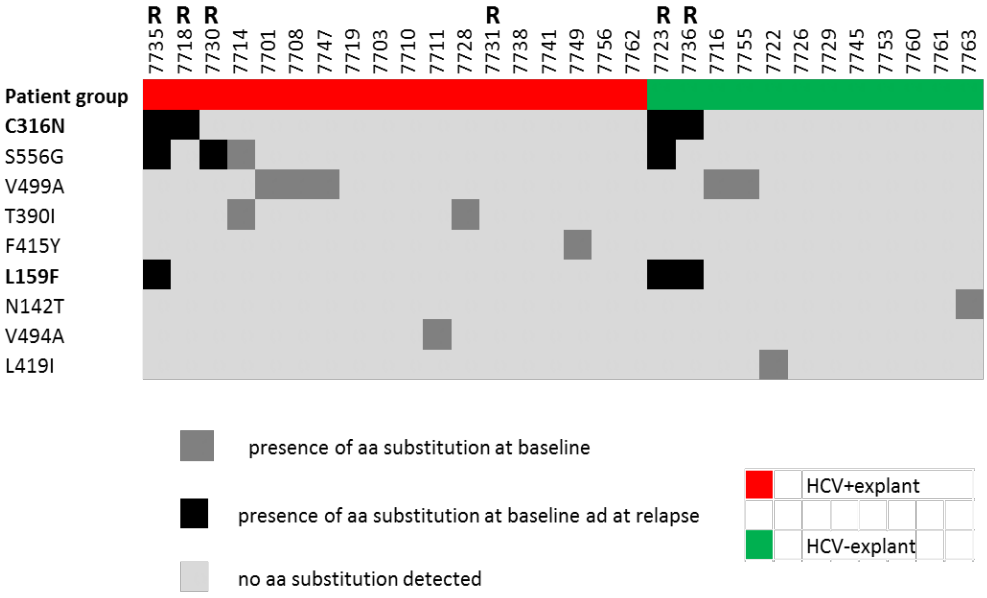

Figure 4. Pattern of specific emergent amino acid substitutions within the NS5B region, at baseline and at time of relapse, by population sequencing and UDPS, present in patients with positive (red group) or negative (green group) HCV-RNA liver explant. Resistance-associated amino acid variants in bold font.

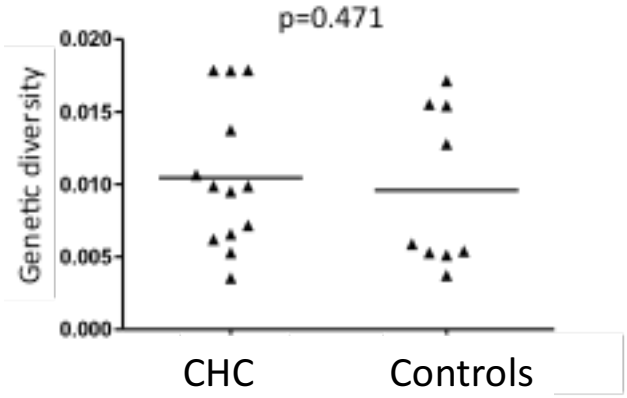

Figure 5a. HCV quasispecies genetic diversity at the moment of liver transplantation (LT), in patients with cholestatic HCV and in the control group.

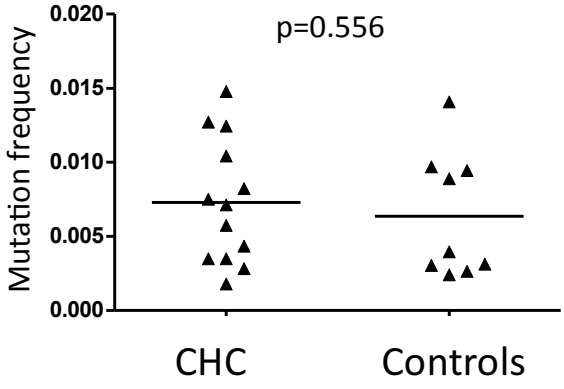

Figure 5b. HCV quasispecies mutation frequency at the moment of liver transplantation (LT), in patients with cholestatic HCV and in the control group.

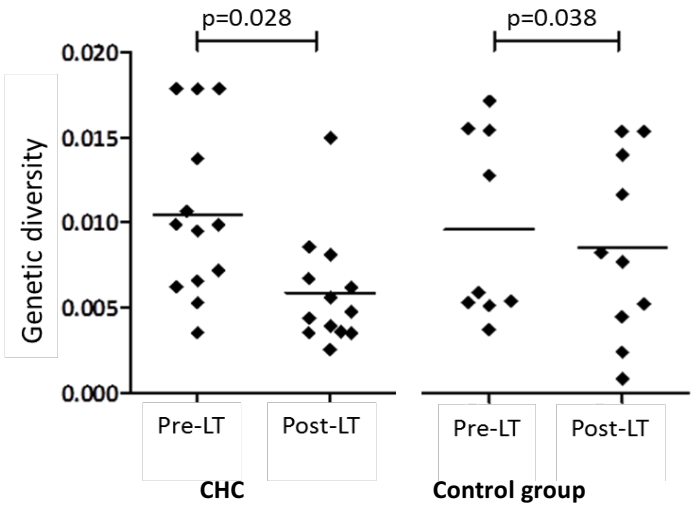

Figure 6. HCV quasispecies genetic diversity before and after liver transplantation (LT), in patients with cholestatic HCV (CHC) and in the control group.

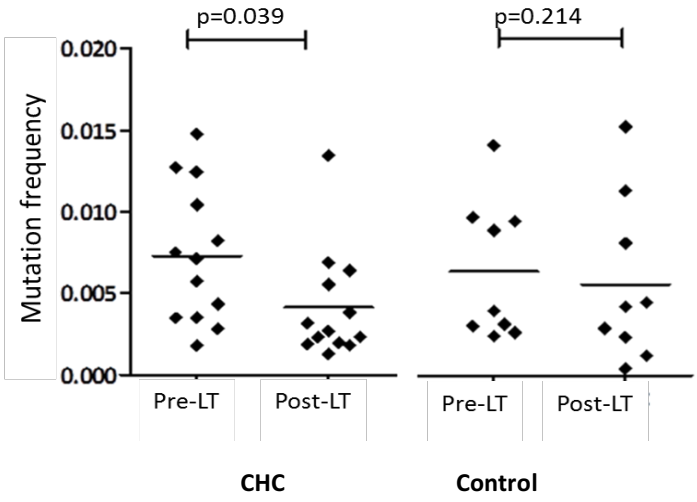

Figure 7. HCV quasispecies mutation frequency before and after liver transplantation (LT), in patients with cholestatic HCV (CHC) and in the control group.

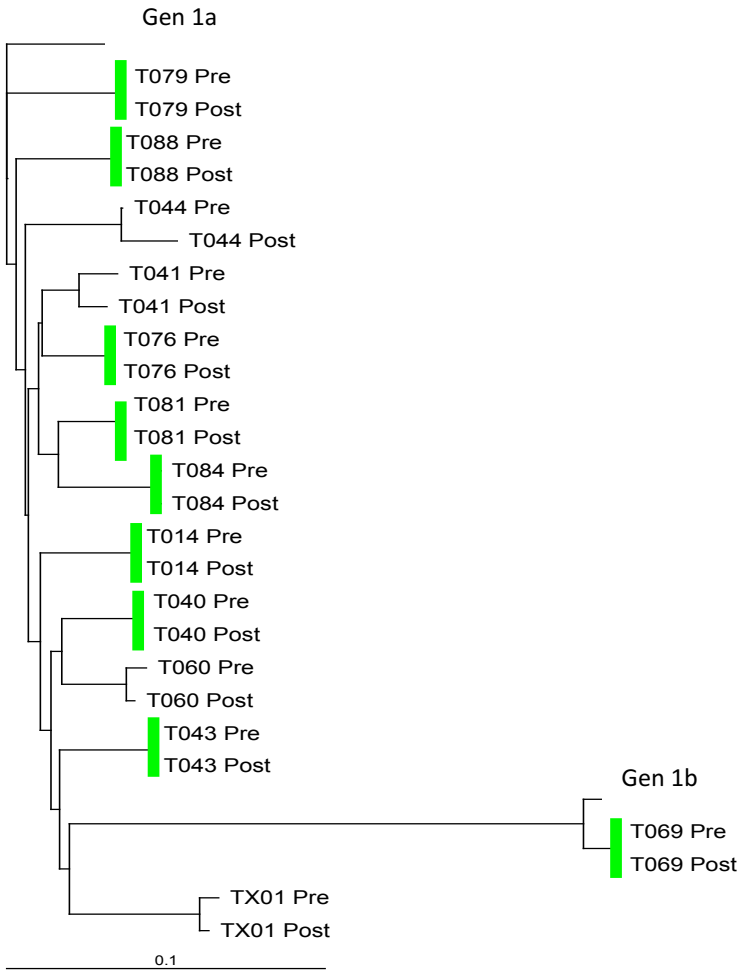

Figure 8a. Phylogenetic tree of NS5B master sequence before and after liver transplantation (LT), in patients with cholestatic HCV

*The green lines indicate those patients with the same master sequence before and after LT.

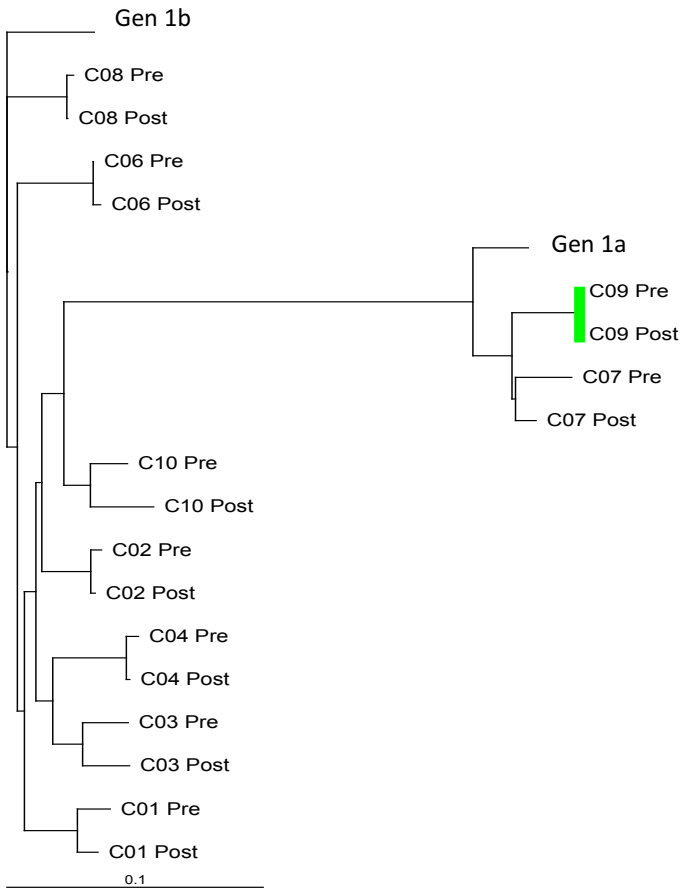

Figure 8b. Phylogenetic tree of NS5B master sequence before and after liver transplantation (LT), in patients with mild HCV recurrence (control group).
*The green lines indicate those patients with the same master sequence before and after LT.

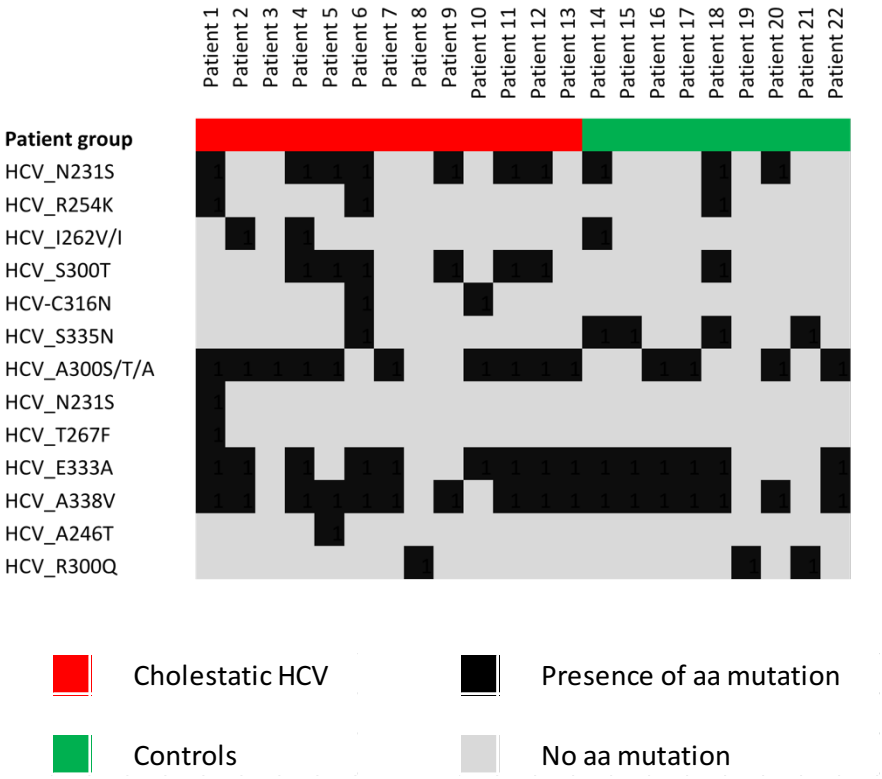

Figure 9. Frequency of mutations present in specific polymorphic sites determined comparing all sequences (pre and post-transplant) of two groups of patients, cholestatic HCV (CHC) and controls.

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