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Hepatitis C virus intrinsic molecular determinants may contribute to the development of cholestatic hepatitis after liver transplantation

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Abstract

Cholestatic hepatitis C (CHC) is a severe form of hepatitis C virus (HCV) infection recurrence that leads to high graft loss rates early after liver transplantation (LT). To investigate the pathogenic mechanisms of CHC, we analysed HCV quasispecies in CHC patients compared to a control group (mild hepatitis C recurrence) by deep pyrosequencing. At the time of LT, NS5B quasispecies complexity was similar between the two groups but, after LT, it decreased more sharply in CHC patients than in the control group. Interestingly, the major variant before LT propagated efficiently and remained as the dominant sequence after LT in 62 % of CHC patients versus 11 % of controls (*P*=0.031). Sequence analysis of the complete non-structural region in a limited number of patients revealed a potential 12 aa signature specific to the CHC group. These data suggest that intrinsic molecular determinants in the circulating HCV quasispecies may provide a fitness advantage, contributing to the development of CHC.

Cirrhosis secondary to chronic hepatitis C infection was the leading indication for liver transplantation (LT) worldwide before the extended use of direct acting antivirals (DAAs). In patients who do not receive DAAs therapy, recurrent hepatitis C virus (HCV) infection of the allograft is universal if the virus is detectable at the moment of transplant surgery. Due to the accelerated course of the disease after LT, if patients are left untreated, almost one-third of liver recipients will progress to cirrhosis within 5 years after LT. Indeed, before the use of DAAs, hepatitis C recurrence after LT led to a significantly reduced graft and patient survival [1–3].

Cholestatic hepatitis C (CHC) is an uncommon and severe variant of HCV infection recurrence that usually occurs early after LT, between 3 and 6 months, in 2–14 % of cases [4–6]. CHC is typically characterized by marked cholestasis and rapid progression to graft failure within the first year after LT. 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 [7]. In CHC patients receiving IFN and ribavirin, the virological response was very low and associated with adverse effects, but introduction of DAAs changed the outcome in these patients. Clinical trials and case repor ts have reported high virological responses with complete recovery of liver function in these patients [8–13]. However, there is still limited understanding of the mechanisms leading to the development of CHC [14]. In general, HCV itself is not considered to be cytopathic, so immune responses against HCV would be the main cause of liver damage in chronic hepatitis C infection [15]. In contrast, hepatocellular injury in CHC might be mediated by a

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Keywords: hepatitis C virus; liver transplantation; cholestatic hepatitis C; quasispecies; deep sequencing.

Abbreviations: CHC, cholestatic hepatitis C; HCV, hepatitis C virus; LT, liver transplantation; VESPA, Viral Epidemiology Signature Pattern Analysis; DAAs, Direct-acting antivirals.

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NS5B deep sequencing data have been deposited in GenBank under accession numbers SAMN10256206–SAMN10256249 (SRA database Bioproject PRJNA493793). N2-NS5B direct sequences have been deposited under accession numbers MK092096–MK092111.

Supplementary material is available with the online version of this article.

Table 1. Clinical and virological characteristics of the patients included in the study

Categorical data are expressed as number (%) and numerical data as median (interquartile range). ALP, alkaline phosphatase; CsA, cyclosporine A; GGT, γ-glutamyltransferase; IS, immunosuppressive.

	Cholestatic HCV (cases), *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 (1b)	12 (92 %)	7 (78 %)	0.368
Recipient IL28B (CC genotype)	1 (7 %)	3 (33 %)	0.091
Main IS drug			0.420
Tacrolimus	9 (70 %)	4 (45 %)	
CsA	2 (15 %)	3 (33 %)	
Everolimus	2 (15 %)	1 (11 %)	
Azathioprine	0 (0 %)	1 (11 %)	
Induction IS therapy	7 (53 %)	1 (11 %)	0.074
Acute rejection	0 (0 %)	3 (33 %)	0.050
Viral load before LT (log IU ml^{-1})	5.65 (4.86–6.17)	5.54 (5.07–5.77)	0.695
Viral load after LT* (log IU ml^{-1})	8.00 (7.63–8.34)	5.69 (5.53–6.40)	0.000
GGT (IU l^{-1})	1513 (765–2708)	216 (23–520)	<0.001
ALP (IU l^{-1})	860 (553–1141)	255 (153–358)	<0.001
Bilirubin (mg dl^{-1})	6.4 (3.5–16)	1.1 (0.6–1.4)	0.001

*Viral load at the time of cholestatic hepatitis C – sample obtained between 1 and 3 months after LT.

cytopathic effect of HCV which, replicating at a high level, could directly induce cellular degeneration in a short period of time, causing progressive and rapid liver failure [16–20].

The analysis of multiple HVR1 clones has shown that the composition of the HCV quasispecies changes after LT [21, 22]. The role of the complexity of viral quasispecies in the pathogenesis of HCV infection recurrence after LT has also been investigated in previous studies, providing controversial results [23–26]. Most of these studies focused on the analysis of the glycoprotein E2, mainly the hypervariable region 1 (HVR1), and used techniques with poor sensitivity, hindering the detection of low-frequency viral populations and limiting the identification of mutations potentially related to the severity of hepatitis C recurrence. Therefore, the analysis of HCV quasispecies composition with more sensitive methodologies may provide a better understanding of CHC.

We hypothesized that genetic evolution of the HCV region coding for the NS5B polymerase, and the selection of specific variants might be implicated in the pathogenesis of CHC, which would explain the high virus replication levels in CHC patients. The aim of our study was to analyse HCV quasispecies in patients with CHC (compared to a control group) by deep pyrosequencing. To this end, we included 22 HCV-infected patients undergoing LT: 13 patients with a diagnosis of CHC and nine patients with mild hepatitis C recurrence (control group). Patients were followed by a standard protocol and relevant variables were collected prospectively and included in a database. This study was approved by the Ethics Committee of the Hospital Clínic of Barcelona and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines and local regulatory requirements. All patients gave written informed consent to participate.

All CHC cases were diagnosed with a liver biopsy after excluding other conditions such as arterial or biliary complications. The diagnosis of CHC was made according to previous published criteria [7]: bilirubin >6 mg dl^{-1}, γ-glutamyl transferase (GGT) and alkaline phosphatase (ALP) ≥5 ULN (Upper Limit of Normal), very high serum HCV-RNA, and typical histology of CHC in the absence of biliary/arterial complications. The characteristic histological state included ballooning of hepatocytes predominantly in the perivenular zone (not necrosis or fallout), paucity of inflammation and variable degrees of cholangiolar proliferation without bile duct loss. Mild hepatitis C recurrence was defined by absent or minimal fibrosis (F0–F1) [27] or liver stiffness measurement below 8.7 kPa, during a follow-up of 5 years after LT. The clinical and virological characteristics of these patients are shown in Table 1. All patients included in the study were infected by HCV genotype 1. In agreement with previous reports [28], CHC patients received organs from older donors than patients with mild recurrent hepatitis C (62 versus 35 years, respectively, *P*=0.003). Recipient age also tended to be higher in CHC patients than in the control group, but it did not reach statistical significance (61 versus 50 years, *P*=0.056). Standard immunosuppressive therapy consisted of steroids plus tacrolimus (TAC) or cyclosporine A (CsA). Although more CHC

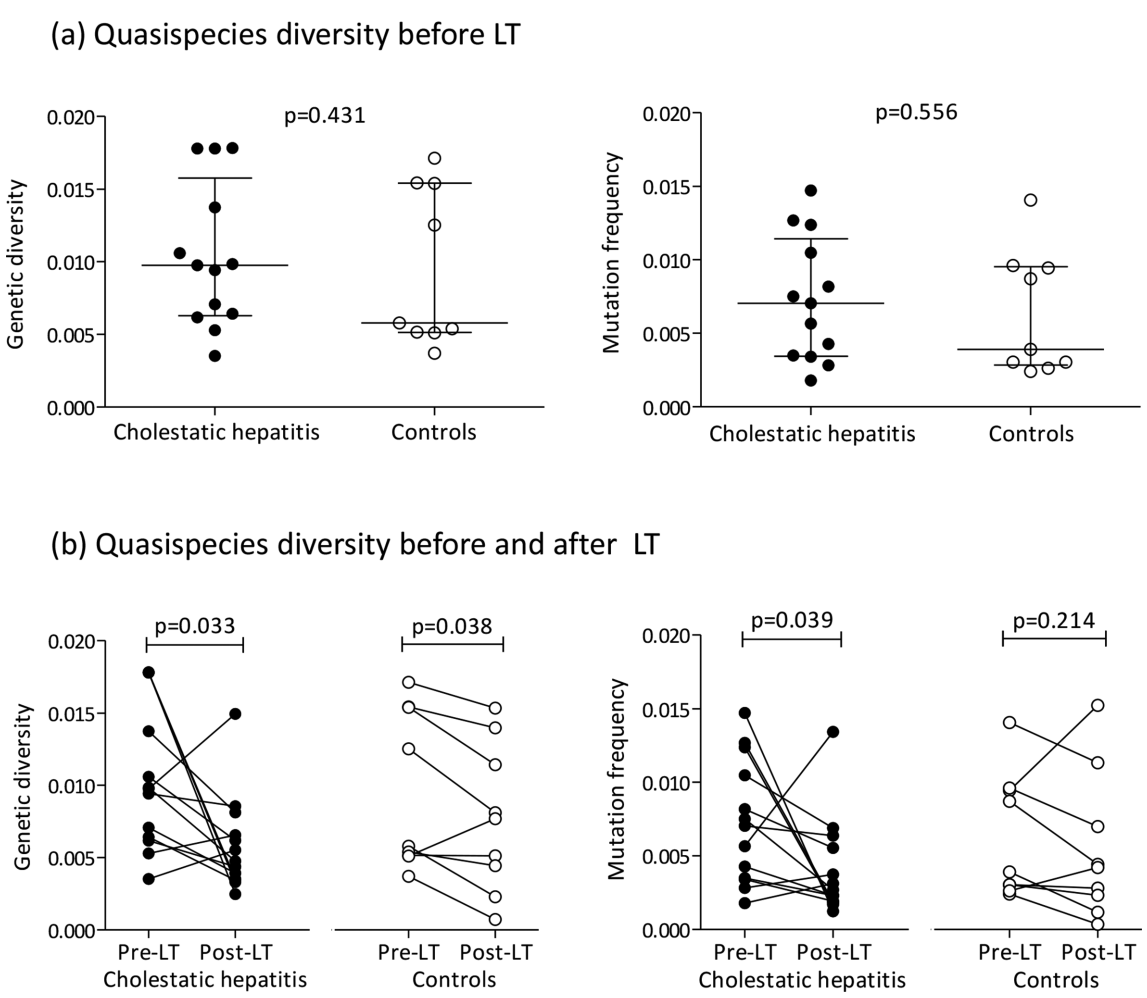

Fig. 1. HCV quasispecies complexity according to the severity of hepatitis C recurrence after LT. NS5B genetic diversity and mutation frequency in CHC patients and in the control group before LT (a), and before and after LT (b). Closed and open circles represent CHC patients and controls, respectively. Solid lines in (a) indicate median values±interquartile range (IQR).

patients (*n*=7, 53 %) received anti-CD25 compared to controls (*n*=1, 11 %), its relevance is difficult to determine due to the small number of patients included in the study. Even so, the few data available on the effects of monoclonal antibody preparations against IL-2 receptor on recurrent hepatitis C after LT are still contradictory [29–31].

Two serum samples were collected from each patient: one sample obtained at the time of LT and another sample obtained between 1 and 3 months after LT at the time of diagnosis of acute hepatitis. We amplified a 340 nt fragment of the NS5B region (nt 8279–8618, according to isolate H77, accession number AF009606). Deep pyrosequencing was performed using the 454/GS-Junior platform (Roche) as previously described [32, 33]. Deep sequencing of the samples yielded a total of 1 163 930 raw reads, 427 881 (37 %) of which accounted for haplotypes that passed the quality filters. Of these, 355 324 reads (31 %) belonged to haplotypes with abundance above 1 % in the respective population, with an average coverage of 8076 reads per sample (Supplementary Material and Table S1, available in the online version of this article) [34, 35]. Viral quasispecies complexity of each sample was computed from these filtered haplotypes by means of nucleotide diversity (Pi, defined as the average number of differences between all possible haplotype pairs corrected based on their frequencies in the population) and the mutation frequency (Mf, the sum of the differences between each haplotype and the dominant haplotype, corrected based on frequency).

At the time of LT, quasispecies diversity and mutation frequency were similar between patients with CHC and patients with mild hepatitis C recurrence (Fig. 1a). After LT, HCV genetic diversity and mutation frequency decreased significantly in CHC patients, showing a marked homogenization of the quasispecies (Pi 0.00976 versus 0.00476, *P*=0.033; and Mf 0.00703 versus 0.00270, *P*=0.039, respectively). In contrast, in the control group, mutation

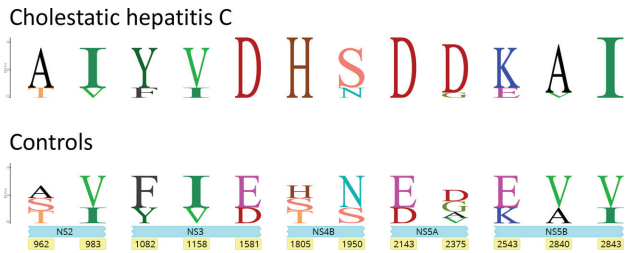

Fig. 2. Identification of specific amino acid motifs associated with the severity of hepatitis C recurrence after LT. Sequence logo illustrating a 12-residue signature that differentiated viral sequences obtained from CHC patients from those derived from the controls. Signature amino acids along the HCV non-structural proteins were identified with VESPA (threshold 0.8). Non-structural proteins are depicted in turquoise and the numbers in the yellow boxes below indicate amino acid positions relative to the Con-1 polyprotein (GenBank accession no. AJ238799).

frequency did not change after LT (Fig. 1b). When we analysed nucleotide diversity among transitions, transversions, and synonymous and non-synonymous sites, we observed that this parameter always decreased after LT for CHC patients but not for controls (data not shown). However, due to the small sample size, this decrease was only statistically significant for transitions (Pi 0.00925 versus 0.00431, P=0.007). We did not find any differences between the number of haplotypes before and after LT in the CHC patients (74 versus 73, P=1.000), but a slight increase in the control group (62 versus 80, P=0.066).

Regarding the propagation of HCV quasispecies after transplantation, the percentage of the master sequence remained constant in the control group (26 versus 28 %, P=0.214), whereas it showed a tendency to increase in the CHC group (27 versus 37 %, P=0.064). Interestingly, in eight of 13 patients with CHC (62 %), the master sequence present at the moment of LT propagated efficiently and remained as the dominant sequence after LT. By contrast, this behaviour was seen in only one of nine patients included in the control group (11 %, P=0.031).

To determine whether specific amino acid motifs were associated with disease outcomes, amino acid master sequences of NS5B were compared between the CHC and control groups by using the Viral Epidemiology Signature Pattern Analysis (VESPA) program with a threshold of 0.8 [36]. We did not find any amino acid signature that differentiated HCV NS5B variants obtained after LT from those in the corresponding pre-LT sample, either for the CHC cases or for the controls. Since there is a high degree of conservation in the NS5B region at the amino acid level, we hypothesized that there might be some molecular determinants related to the severity of the hepatitis C recurrence in other regions, apart from the NS5B polymerase. Thus, we amplified and analysed by direct sequencing a fragment of 6321 bp (2107 aa) encompassing almost the complete non-structural region, from NS2 to NS5B (2916–9236 bp) in a selected group of genotype 1b infected patients: five CHC and three controls (Supplementary Material). Similarly to the previous NS5B analysis, we did not find any signature pattern differentiating viral sequences obtained before or after LT, for either of the two groups of patients. Interestingly, by applying VESPA with a threshold of 0.8, we found a 12-residue signature that differentiated viral sequences obtained from CHC patients from those derived from the controls. As shown in Fig. 2, variants carrying D1581 (helicase domain of NS3), H1805 (NS4B), D2143 (NS5A) and I2843 (NS5B polymerase) were detected in the five CHC patients analysed. This suggests that some of these positions may confer a fitness advantage which would account for the high viral loads observed in these patients. Nonetheless, we cannot rule out that there may be variations in the frequency of amino acid residues in other positions in the same variant as a result of hitchhiking selection.

Before the use of DAAs, CHC was the most severe form of HCV infection recurrence after LT. Because of its relatively low frequency, limited clinical data are available, and the risk factors for CHC development remain unresolved [14]. Previous reports have shown that older donors [28], corticosteroid treatment for acute cellular rejection [37], high levels of HCV RNA after LT [38] and IL28B genotype [39] may be implicated in the development of CHC. To our knowledge, this is the first study that has investigated HCV quasispecies evolution in patients with CHC after LT based on deep sequencing. We have shown that the diversity of HCV quasispecies at the time of LT did not correlate with disease progression after LT. On the contrary, after LT, patients with CHC showed a pronounced homogenization of the HCV quasispecies that was not observed in patients with mild hepatitis C recurrence. Our data are in agreement with previous studies suggesting an inverse correlation between HCV genetic diversification and the severity of hepatitis C recurrence during the first year after LT [24, 25, 40]. Several authors have previously reported that quasispecies complexity decreases in the absence of an effective immune response early after LT [21, 26, 41, 42]. However, this has not been confirmed by others [23, 25]. These

conflicting results may be due, in part, to the HCV region analysed (envelope glycoprotein E2, mainly HVR1), the different time points at which the post-LT serum samples were obtained, the characteristics of the study populations (sometimes poorly defined) and the techniques available at that time, such as single-strand conformation polymorphism (SSCP), heteroduplex mobility assay (HMA) and sequencing of a limited number of clones, which are far less sensitive methods than deep sequencing.

Our results suggest that among the divergent genomes present in the viral quasispecies before LT, one or a few viruses may infect the graft, overgrow the others and generate a relatively uniform quasispecies after LT. One could argue that the low-grade genetic evolution of NS5B observed in CHC might be related to the weak immune pressure against HCV, resulting from the immunosuppressive therapy. However, we did not observe any significant difference between the two groups in terms of immunosuppression regimens. On the other hand, the fact that lymphocytes from patients with severe hepatitis C recurrence fail to mount virus-specific T-cell responses [43] suggests that some intrinsic features in the viral genome might play a role in the pathogenesis of HCV-related liver injury after LT. Supporting the latter suggestion, we found that the master sequence present before LT propagated in most of our CHC patients after LT, but not in patients with mild HCV recurrence. Similarly, Sullivan *et al.* [24] demonstrated that quasispecies major variants present in pre-transplant serum were efficiently propagated after LT in three patients with severe HCV recurrence but not in the two patients with mild recurrence. In addition, we also identified a set of 12 aa motifs along the non-structural domain that distinguished viral genomes infecting CHC patients from the controls. Although none of the majority of the amino acids in the signature was exclusive of each group of patients, four signature sites were found in all the CHC sequences but only in 33 % of the controls. However, because this analysis was only performed in five CHC and three control patients, these results should be taken with caution unless they are validated in a larger number of patients and/or *in vitro* using the HCV subgenomic replicon system.

The main limitations of our study are the small sample size due to the low incidence of this uncommon complication, and the fact that, today, CHC patients can be safely treated with DAAs, which clearly lessens its clinical impact in the setting of LT. However, unlike immune-mediated injury caused by chronic hepatitis C, CHC represents a unique model to investigate virus-induced direct liver cell damage and, thus, to decipher the mechanisms involved in the pathogenesis of this specific virological entity.

In conclusion, our data suggest that some of the HCV variants infecting the graft may carry molecular determinants that might confer a higher pathogenic potential. Whether ineffective immune responses allow the propagation of more cytopathic variants, or whether viral determinants present in pathogenic variants are able to suppress or evade immune control remain to be elucidated.

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Conflicts of interest
XF received unrestricted gran support from Abbvie and has acted as advisor for Abbvie and Gilead.

Ethical statement
This study was approved by the Ethics Committee of the Hospital Clínic of Barcelona and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines and local regulatory requirements. All patients signed a written informed consent to participate.

References

1. **Gane EJ.** The natural history of recurrent hepatitis C and what influences this. *Liver Transpl* 2008;14:S36–S44.
2. **Crespo G, Mariño Z, Navasa M, Forns X.** Viral hepatitis in liver transplantation. *Gastroenterology* 2012;142:1373–1383.
3. **Berenguer M, Schuppan D.** Progression of liver fibrosis in post-transplant hepatitis C: mechanisms, assessment and treatment. *J Hepatol* 2013;58:1028–1041.
4. **Narang TK, Ahrens W, Russo MW.** Post-liver transplant cholestatic hepatitis C: a systematic review of clinical and pathological findings and application of consensus criteria. *Liver Transpl* 2010;16:1228–1235.
5. **Dixon LR, Crawford JM.** Early histologic changes in fibrosing cholestatic hepatitis C. *Liver Transpl* 2007;13:219–226.
6. **Xiao SY, Lu L, Wang HL.** Fibrosing cholestatic hepatitis: clinicopathologic spectrum, diagnosis and pathogenesis. *Int J Clin Exp Pathol* 2008;1:396–402.
7. **Wiesner RH, Sorrell M, Villamil F.** Report of the first International Liver Transplantation Society expert panel consensus conference on liver transplantation and hepatitis C. *Liver Transpl* 2003;9:S1–S9.
8. **Forns X, Mutimer D, Manns M, Reddy KR, Everson GT *et al.*** P0779 : Ledipasvir/sofosbuvir with ribavirin for the treatment of fibrosing cholestatic hepatitis C after liver transplantation. *J Hepatol* 2015;62:S623.
9. **Wadhawan M, Vij V, Makki K, Bansal N, Kumar A.** Early acute severe HCV recurrence after transplantation: from universal mortality to cure. *J Clin Exp Hepatol* 2017;7:28–32.
10. **Manns M, Samuel D, Gane EJ, Mutimer D, McCaughan G *et al.*** Ledipasvir and sofosbuvir plus ribavirin in patients with genotype 1 or 4 hepatitis C virus infection and advanced liver disease: a multicentre, open-label, randomised, phase 2 trial. *Lancet Infect Dis* 2016;16:685–697.
11. **Leroy V, Dumortier J, Coilly A, Sebagh M, Fougerou-Leurent C *et al.*** Efficacy of sofosbuvir and daclatasvir in patients with fibrosing cholestatic hepatitis C after liver transplantation. *Clin Gastroenterol Hepatol* 2015;13:1993–2001.
12. **Issa D, Eghtesad B, Zein NN, Yerian L, Cruise M *et al.*** Sofosbuvir and simeprevir for the treatment of recurrent hepatitis C with

fibrosing cholestatic hepatitis after liver transplantation. *Int J Organ Transplant Med* 2016;7:38–45.

13. Forns X, Charlton M, Denning J, Mchutchison JG, Symonds WT *et al.* Sofosbuvir compassionate use program for patients with severe recurrent hepatitis C after liver transplantation. *Hepatology* 2015;61:1485–1494.
14. Mccaughan GW, Bowen DG. Pathogenesis of cholestatic hepatitis C. *J Hepatol* 2011;54:392–394.
15. Ramírez S, Pérez-del-Pulgar S, Forns X. Virology and pathogenesis of hepatitis C virus recurrence. *Liver Transpl* 2008;14:S27–S35.
16. Lim HL, Lau GK, Davis GL, Dolson DJ, Lau JY. Cholestatic hepatitis leading to hepatic failure in a patient with organ-transmitted hepatitis C virus infection. *Gastroenterology* 1994;106:248–251.
17. Zylberberg H, Carnot F, Mamzer MF, Blancho G, Legendre C *et al.* Hepatitis C virus-related fibrosing cholestatic hepatitis after renal transplantation. *Transplantation* 1997;63:158–160.
18. Delgado J, Muñoz de Bustillo E, Ibarrola C, Colina F, Morales JM *et al.* Hepatitis C virus-related fibrosing cholestatic hepatitis after cardiac transplantation: is azathioprine a contributory factor? *J Heart Lung Transplant* 1999;18:607–610.
19. Demetris AJ. Evolution of hepatitis C virus in liver allografts. *Liver Transpl* 2009;15 Suppl 2:S35–S41.
20. Giard JM, Terrault NA. Severe Cholestatic Hepatitis C in Transplant Recipients: No Longer a Threat to Graft Survival. *Clin Gastroenterol Hepatol* 2015;13:2002–2004.
21. Feliu A, Gay E, García-Retortillo M, Saiz JC, Forns X. Evolution of hepatitis C virus quasispecies immediately following liver transplantation. *Liver Transpl* 2004;10:1131–1139.
22. Ramirez S, Perez-del-Pulgar S, Carrion JA, Costa J, Gonzalez P *et al.* Hepatitis C virus compartmentalization and infection recurrence after liver transplantation. *Am J Transplant* 2009;9:1591–1601.
23. Pessoa MG, Bzowej N, Berenguer M, Phung Y, Kim M *et al.* Evolution of hepatitis C virus quasispecies in patients with severe cholestatic hepatitis after liver transplantation. *Hepatology* 1999;30:1513–1520.
24. Sullivan DG, Wilson JJ, Carithers RL, Perkins JD, Gretch DR. Multigene tracking of hepatitis C virus quasispecies after liver transplantation: correlation of genetic diversification in the envelope region with asymptomatic or mild disease patterns. *J Virol* 1998;72:10036–10043.
25. Sánchez-Fueyo A, Giménez-Barcons M, Puig-Basagoiti F, Rimola A, Sánchez-Tapias JM *et al.* Influence of the dynamics of the hypervariable region 1 of hepatitis C virus (HCV) on the histological severity of HCV recurrence after liver transplantation. *J Med Virol* 2001;65:266–275.
26. Gretch DR, Polyak SJ, Wilson JJ, Carithers RL, Perkins JD *et al.* Tracking hepatitis C virus quasispecies major and minor variants in symptomatic and asymptomatic liver transplant recipients. *J Virol* 1996;70:7622–7631.
27. Scheuer PJ. The nomenclature of chronic hepatitis: time for a change. *J Hepatol* 1995;22:112–114.
28. Verna EC, Abdelmessih R, Salomao MA, Lefkowitch J, Moreira RK *et al.* Cholestatic hepatitis C following liver transplantation: an outcome-based histological definition, clinical predictors, and prognosis. *Liver Transpl* 2013;19:78–88.
29. Nelson DR, Soldevila-Pico C, Reed A, Abdelmalek MF, Hemming AW *et al.* Anti-interleukin-2 receptor therapy in combination with mycophenolate mofetil is associated with more severe hepatitis C recurrence after liver transplantation. *Liver Transpl* 2001;7:1064–1070.
30. Calmus Y, Scheele JR, Gonzalez-Pinto I, Jaurrieta EJ, Klar E *et al.* Immunoprophylaxis with basiliximab, a chimeric anti-interleukin-2 receptor monoclonal antibody, in combination with azathioprine-containing triple therapy in liver transplant recipients. *Liver Transpl* 2002;8:123–131.
31. Neuhaus P, Clavien PA, Kittur D, Salizzoni M, Rimola A *et al.* Improved treatment response with basiliximab immunoprophylaxis after liver transplantation: results from a double-blind randomized placebo-controlled trial. *Liver Transpl* 2002;8:132–142.
32. Quer J, Gregori J, Rodríguez-Frias F, Buti M, Madejon A *et al.* High-resolution hepatitis C virus subtyping using NS5B deep sequencing and phylogeny, an alternative to current methods. *J Clin Microbiol* 2015;53:219–226.
33. Pérez-del-Pulgar S, Gregori J, Rodríguez-Frías F, González P, García-Cehic D *et al.* Quasispecies dynamics in hepatitis C liver transplant recipients receiving grafts from hepatitis C virus infected donors. *J Gen Virol* 2015;96:3493–3498.
34. Gregori J, Esteban JI, Cubero M, Garcia-Cehic D, Perales C *et al.* Ultra-deep pyrosequencing (UDPS) data treatment to study amplicon HCV minor variants. *PLoS One* 2013;8:e83361.
35. Gregori J, Salicrú M, Domingo E, Sanchez A, Esteban JI *et al.* Inference with viral quasispecies diversity indices: clonal and NGS approaches. *Bioinformatics* 2014;30:1104–1111.
36. Korber B, Myers G. Signature pattern analysis: a method for assessing viral sequence relatedness. *AIDS Res Hum Retroviruses* 1992;8:1549–1560.
37. Berenguer M, Aguilera V, Prieto M, San Juan F, Rayón JM *et al.* Significant improvement in the outcome of HCV-infected transplant recipients by avoiding rapid steroid tapering and potent induction immunosuppression. *J Hepatol* 2006;44:717–722.
38. Doughty AL, Spencer JD, Cossart YE, Mccaughan GW. Cholestatic hepatitis after liver transplantation is associated with persistently high serum hepatitis C virus RNA levels. *Liver Transpl Surg* 1998;4:15–21.
39. Hanouneh IA, Zein NN, Askar M, Lopez R, John B. Interleukin-28B polymorphisms are associated with fibrosing cholestatic hepatitis in recurrent hepatitis C after liver transplantation. *Clin Transplant* 2012;26:E335–E336.
40. Massaguer A, Ramírez S, Carrión JA, González P, Sánchez-Tapias JM *et al.* Evolution of the NS3 and NS5B regions of the hepatitis C virus during disease recurrence after liver transplantation. *Am J Transplant* 2007;7:2172–2179.
41. Martell M, Esteban JI, Quer J, Vargas V, Esteban R *et al.* Dynamic behavior of hepatitis C virus quasispecies in patients undergoing orthotopic liver transplantation. *J Virol* 1994;68:3425–3436.
42. Yun Z, Barkholt L, Sönnerborg A. Dynamic analysis of hepatitis C virus polymorphism in patients with orthotopic liver transplantation. *Transplantation* 1997;64:170–172.
43. Rosen HR, Hinrichs DJ, Gretch DR, Koziel MJ, Chou S *et al.* Association of multispecific CD4(+) response to hepatitis C and severity of recurrence after liver transplantation. *Gastroenterology* 1999;117:926–932.