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Effect of scavenger receptor BI antagonist ITX5061 in patients with hepatitis C virus infection undergoing liver transplantation

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Abbreviations: AOC, area over the curve; BMI, body mass index; DAA, directly acting antiviral; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDL, high density lipoprotein; HVR, hypervariable region; IQR, interquartile range; MELD, model for end-stage liver disease; PCR, polymerase chain reaction; SR-BI, scavenger receptor B-I; UDPS, ultradeep pyrosequencing.

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Abstract

Hepatitis C virus (HCV) entry inhibitors have been hypothesized to prevent infection of the liver after transplantation. ITX5061 is a Scavenger Receptor B-I (SR-BI) antagonist that blocks HCV entry and infection *in vitro*. We assessed the safety and efficacy of ITX5061 to limit HCV infection of the graft. The study included 23 HCV infected patients undergoing liver transplantation. The first 13 "control" patients did not receive drug. The subsequent 10 patients received ITX5061 150 mg immediately pre- and post-transplant, and daily for 1 week thereafter. ITX5061 pharmacokinetics and plasma HCV RNA were quantified. Viral genetic diversity was measured by ultradeep pyrosequencing. ITX5061 was well tolerated with measurable plasma concentrations during therapy. Whilst the median HCV RNA reduction was greater in ITX treated patients at all time points in the first week after transplantation there was no difference in the overall change in the area over the HCV RNA curve in the 7-day treatment period. However, in genotype 1 infected patients treatment was associated with a sustained reduction in HCV RNA levels compared to the control group (area over the HCV RNA curve analysis, p=0.004). Ultradeep pyrosequencing revealed a complex and evolving pattern of HCV variants infecting the graft during the first week. ITX5061 significantly limited viral evolution where the median divergence between day 0 and day 7 was 3.5% in the control group compared to 0.1% in the treated group. **Conclusions:** ITX5061 reduces plasma HCV RNA post transplant notably in genotype 1 infected patients and slows viral evolution. Following liver transplantation the likely contribution of extrahepatic reservoirs of HCV necessitates combining entry inhibitors such as ITX5061 with inhibitors of replication in future studies. *Clinicaltrials.gov NCT01292824*.

Introduction

Hepatitis C virus (HCV) infection is a global health problem with more than 170 million people infected worldwide. Chronic infection with HCV can lead to cirrhosis and hepatocellular carcinoma (HCC) and is a leading indication for liver transplantation [1]. At the time of transplantation HCV circulating in the periphery infects the newly transplanted graft [2-4] and can lead to progressive liver injury. HCV is one of the major causes of graft failure [5-7] and preventing infection of the graft is a rational strategy to combat that complication.

Studies investigating the use of polyclonal anti-HCV immunoglobulins or anti-HCV monoclonal antibodies to prevent HCV reinfection of the newly transplanted liver have met with limited success [8, 9]. However, recent reports detailing the passive transfer of the neutralizing human monoclonal antibody MBL-HCV1 showed some protection in humans and chimpanzee [10, 11], illustrating the potential of this approach. The major cell type supporting HCV replication in the liver is the hepatocyte and infection is defined by four essential host entry factors: CD81, scavenger receptor B-I (SR-BI), claudin-1, and occludin (reviewed in [12, 13]). Recent studies showing that antibodies targeting SR-BI limit HCV infection of humanized mice highlight a role for SR-BI antagonists to prevent HCV infection of the newly transplanted liver [14, 15].

SR-BI is a receptor for high-density lipoprotein (HDL) and is an essential component of the reverse cholesterol transport system [16]. ITX5061 was originally developed as a p38 mitogen-activated protein kinase inhibitor and was evaluated in early phase clinical trials for treating rheumatoid arthritis and psoriasis where treated subjects showed an elevation in serum HDL (iTherX, data on file). Mechanistic studies identified ITX5061 as an antagonist of SR-BI [17] that could inhibit HCV entry *in vitro* and was effective against diverse viral genotypes [18]. More recently ITX5061 was reported to act additively with interferon, ribavirin and the protease inhibitor telaprevir *in vitro* and, given its mode of action, no cross-resistance is expected with any of the direct acting antivirals (DAAs) currently in development [19].

We assessed the safety and efficacy of ITX5061 to limit HCV infection of the graft. Viral RNA levels were measured in treated and untreated patients during the post-transplant period and viral evolution assessed by ultradeep pyrosequencing (UDPS).

Patients and Methods

Study design. An open label phase Ib study was designed to assess the effect of ITX5061 in patients undergoing liver transplantation at a single centre (Queen Elizabeth Hospital Birmingham, UK). All patients gave informed consent and ethical approval was given by the UK National Research Ethics Service (reference 10/H0301/36). Patients were allocated sequentially to a no treatment control group or to treatment with ITX5061, 150 mg/day via the enteral route for 1 week. Treatment duration was determined with reference to the known safety profile of ITX5061 in patients without liver disease. Although it was intended that 10 subjects would be enrolled into each group, an interim analysis following the enrolment of the first 5 patients suggested that more detailed HCV kinetic monitoring would provide a more robust baseline of viral kinetics in the untreated patients. The control group was therefore increased to 13 subjects. The study was registered at clinicaltrials.gov (NCT01292824).

Population. The study enrolled men and women between the ages of 18 and 65 years who were suitable for liver transplantation. Subjects with HCV associated end-stage liver disease or HCC were enrolled regardless of their infecting genotype or previous anti-viral treatment. Subjects co-infected with HBV or HIV were excluded, as were patients receiving a liver from a HCV positive donor.

Study drug. ITX5061 was formulated as a 25 mL solution for oral or nasogastric use containing 150 mg drug in a vehicle containing 20% (w/w) hydroxypropyl-beta-cyclodextrin in 10 mM aqueous citric acid. A dose of 150 mg was selected following pre-clinical studies predicting a 10-fold excess over the EC_{90} for inhibiting HCV entry [18]. Dosing at 150 mg was further supported by studies conducted in the initial development of ITX5061 where this dose was sufficient to block uptake of HDL (the physiological ligand of SR-BI) as evidenced by increased serum HDL levels in treated study participants [17]. The first dose was administered orally approximately 1 hour before the induction of anaesthesia. A second dose was given via a nasogastric tube on arrival to the intensive care unit following liver transplantation and then once daily for 7 days thereafter.

Pharmacokinetics. Plasma ITX5061 concentrations were measured by liquid chromatography/mass spectrometry [20]. Since ITX5061 is primarily metabolised in the liver an interim analysis of ITX5061 plasma concentrations was performed on the first 3 treated subjects. Review of these data by the trial steering group and by the Medicines and Health Regulatory Authority UK, recommended continued enrolment and treatment of the remaining 7 patients.

HCV replication kinetics. Plasma was collected at screening, before surgery, at the time of transplantation, and during a follow up period of 90 days. HCV RNA levels were measured on admission to hospital, immediately following the induction of anaesthesia, at the time of portal vein clamping (the start of the anhepatic phase), immediately before perfusion of the allograft, and an hour later. Plasma samples were collected every 4 hours during the first post-transplant day, daily for the first week, weekly for the first month, and monthly thereafter up to 90 days. Plasma HCV RNA was measured using the COBAS TaqMan HCV Test v.2.0 in a Health Protection Agency UK accredited laboratory.

Viral sequencing. HCV RNA was purified from plasma obtained immediately before surgery and 7 days later. Each sample was analysed by UDPS of the viral structural genes (core, E1, E2 and P7) including the hypervariable region (HVR) using genotype specific primers (**Suppl. Table 1)**. Amplicons were ligated to adaptors (Nextera Tagmentation), amplified by emulsion polymerase chain reaction (PCR) and sequenced on a 454 GS Junior (Roche). The raw sequence outputs ("reads") were assembled using the Assemble Viral 454 [21] and VICUNA *de novo* assembler software [22] to form a consensus assembly. The reads were corrected for systematic 454 errors and aligned to the consensus assembly using the ReadClean 454 and V-Phaser algorithms [23]. Average sequence lengths varied from 342 to 405 nucleotides and on average 3900 reads were generated for each sample, a total of 1·5 to 2·9 x 10^6 bases and an average coverage of 350 to 500 reads for each base. Heat-maps of the viral envelope (E2) region were generated to graphically represent sequence polymorphisms. Genetic diversity within samples, and divergence between samples were assessed by calculating genetic distance estimates. Pairwise comparisons of sequences allowed estimates of genetic diversity of viral quasispecies before and after therapy.

Statistics. The primary endpoint of this study was to assess ITX5061 safety in liver transplant recipients. Adverse events were graded in accordance with the National Cancer Institute Common Terminology Criteria version 4.0 and were tabulated according to treatment allocation. The secondary endpoint was to measure plasma HCV RNA levels in treated and untreated patients. As a global summary of HCV RNA changes in the first week after transplantation the area over the curve (AOC) was calculated from the anhepatic baseline value in line with previous similar studies [9]. Categorical data were compared using Fisher's exact test whilst continuous data were compared using the Mann-Whitney test. Analyses were performed using Prism v 6.0e (Graphpad) and CLC 6.1 workbench.

Role of the funding source. Representatives of iTherX Pharma Inc. were involved in the initial design of the study. None of the other funding agencies had any role in study conduct. The corresponding author had full access to the study data and made the final decision to submit for publication.

Results

Patients. 23 patients were included in the study (**Fig.1**) and their characteristics are listed in **Table 1**. The patients were predominantly males (21/23) infected with HCV genotype 1 (13 cases) or genotype 3 (8 cases). Sixteen patients were transplanted for liver failure and seven for HCC. The baseline median HCV RNA load was 5.8 $\log_{10}$ IU/mL in in the untreated group and 5.3 $\log_{10}$ IU/mL in the ITX5061 treated group. There were no significant differences in any of the clinical parameters between the groups. All transplanted livers were from deceased donors and the donor age was comparable between the groups.

Safety. During the study period there were a total of 165 serious adverse events (SAE) and 342 adverse events. There was no difference in the frequency or severity of adverse events between control or treatment groups (**Table 2**) and no adverse events leading to the discontinuation of ITX5061. On a per patient basis the median number of grade 1 and 2 adverse events was 15 in the untreated group and 14 in the ITX5061 treated group (p=0.96, Mann-Witney test). Similarly the median number of SAEs was 7 in the untreated group compared with 6 in the ITX treated group (p=0.45, Mann-Witney test). One patient in the control group died from a subarachnoid haemorrhage 8 weeks after transplantation. A further patient required emergency re-transplantation for haemorrhagic necrosis at day 8 after transplantation. The remaining SAEs were related to elevations of transaminases >2000 IU/mL, hyperglycaemia, symptomatic anaemia, acute kidney injury, severe infection, agitation and confusion, and perioperative hypotension associated with ventricular tachycardia. For one treated patient, elevated transaminases were prolonged and initially categorised as “probably related to ITX5061”. Treatment was continued and transaminases declined in line with other treated and untreated patients. The peak transaminase levels measured 24 hours following transplantation were comparable between the groups (**Suppl. Fig.1**). There was no difference in trough plasma tacrolimus concentrations measured at day 3, day 5, and day 7 after transplantation although concentrations at day 7 were numerically lower (5.4 vs. 7.2 µg/L, **Table 3**).

Pharmacokinetics. Plasma ITX5061 concentrations were measured during the treatment period in all patients. Oral administration approximately 1 hour before transplantation resulted in good absorption and median plasma concentrations of 336 ng/mL (interquartile range, 291 – 481ng/mL) at the start of anaesthesia (**Suppl. Fig.2**). At the time of reperfusion plasma drug levels were 5- to 90-fold in excess of the predicted EC_{90} (3 ng/mL) to limit HCV entry [18]. During the early post operative period there was no evidence of drug accumulation and trough levels (24±4 hours since last dose) were, on average, greater than 18-fold in excess of the predicted EC_{90} in all subjects.

HCV RNA kinetics after transplantation. We observed a rapid decrease in HCV RNA immediately after allograft reperfusion in all patients (**Fig.2A**). The initial decline in the first 16 hours was similar in both groups, suggesting that the drug has minimal effect on viral clearance. We noted a rebound of viral RNA 24 hours post surgery in the untreated patients that most likely reflects de novo infection and release of viral particles into the periphery. Importantly, this rebound was not apparent in any patient receiving ITX5051 (**Fig.2B**), suggesting that ITX5061 limits primary infection of the allograft.

Two patterns of HCV RNA replication kinetics were observed in the untreated group between 24 and 168 hours after transplantation: 5 patients showed an early increase in HCV RNA (>10 fold) and the remaining 8 showed stable or decreasing viral loads. The majority of subjects in the ITX5061 treated group (7/10) showed stable or decreasing viral loads. Two treated patients showed >10 fold increase in HCV RNA between 24 and 72 hours after transplant and a third patient showed increasing HCV RNA between 4 and 7 days (**Fig.3A**). The median HCV RNA decline was greater in ITX5061 treated patients throughout the treatment period although there was no significant difference when the AOC was calculated (**Fig.3B**). At one month post transplantation, HCV RNA levels were comparable in both groups (**Fig.3C&D**). The majority of patients were infected with HCV genotype 1 and we assessed the effect of ITX5061 on HCV RNA kinetics during the first week of this sub-group. The median reduction in HCV RNA was greater in the ITX5061 treated group: no patients in this group showed an increase in HCV RNA (**Fig.4A&B**).

HCV quasispecies analysis. To assess whether ITX5061 exerts selective pressure the viral quasispecies in the plasma on the day of transplant and at the end of therapy were compared. A heatmap of E2 diversity shows the HVR to be the most variable region sequenced and provides an indicator of genetic diversity (**Fig.5A**) [24]. Two patterns of quasispecies diversity were observed at baseline: in the untreated group (median E2 diversity 9.3%) four patients showed a high level of diversity, with >10 HVR sequence variants at day 0 (**Fig.5A upper, and Fig.5B**); the remaining 5 patients had significantly fewer variants (2-3) at day 0 (**Fig.5A lower, and Fig.5B**). Among the treated group (median E2 diversity 10.2%) similar patterns were seen, with four patients showing high variability and 4 lower (**Fig.5B**). We failed to observe an association between baseline viral diversity and replication kinetics in either group (**Fig.5B**).

Sequencing the circulating virus after 7 days showed that in both untreated and treated patients, E2 diversity was preserved (median diversity 10.4% and 11.6% respectively). Analysis of the sequences in the untreated patients showed that, irrespective of their baseline sequence diversity, after 7 days many of the initial sequences were replaced by a new group of variants. In contrast, the majority of treated patients showed no change in their viral sequence profiles, with new HVR variants being detected in only two treated patients. The majority of treated subjects had exactly the same HVR sequences at 0 and 7 days, albeit with some alteration in their relative abundance. The average pairwise change in the HVR was significantly higher in the untreated compared to the ITX5061 treated patients (control patient median change 3.5%, ITX5061 treated 0.1%, Mann-Whitney p=0.027) (**Fig.5C**). Remarkably, for one treated patient not one polymorphic residue was observed among the 1,152 HVR sequences obtained at day 7. We observed a similar change when the entire E2 region was analysed, however, this was not statistically significant (data not shown). These observations show that ITX5061 restricts HCV evolution.

Discussion

HCV infects the newly transplanted liver and can cause graft injury with up to 25% of recipients experiencing graft failure 10 years post transplant, highlighting the clinical relevance of preventing infection of the allograft. The SR-BI antagonist ITX5061 was well tolerated and the drug limited HCV RNA rebound 24 hours post surgery. This was sustained most notably in patients infected with genotype 1 viruses. Furthermore, ITX5061 limited HCV evolution suggesting that the drug reduces *de novo* infection events that drive the production of new viral variants.

The safety profile of ITX5061 was previously established in patients with autoimmune diseases (at doses up to 300 mg) and in chronic HCV infected patients with no indications for liver transplantation. This was a "first in indication" study to determine safety in the liver transplant population where additional issues of surgery and concomitant medications apply. We did not identify any excess of adverse events in the treated group and there was no evidence for a significant drug interaction with tacrolimus. Tacrolimus trough concentrations were numerically lower in the ITX5061 treated group at day 7 following transplantation and larger studies would be necessary to determine both safety and the presence or absence of significant drug-drug interactions with a greater degree of accuracy. Importantly there was no deleterious accumulation of ITX5061 in the plasma due to impaired hepatic clearance in the hours and days following transplantation although the majority of patients included in this study had relatively well preserved synthetic function and low median model for end-stage liver disease (MELD) scores. Whilst there is no reason to expect greater toxicity in patients with more advanced liver failure since treatment targets the allograft, continued assessment of drug levels with pharmacokinetic analyses would be appropriate in future studies.

This trial is the first to target a host entry factor in HCV infected subjects undergoing liver transplantation. In a similar study Chung and colleagues showed that a humanised antibody targeting the HCV encoded E2 envelope glycoprotein reduced viral RNA burden in 6 subjects in an analogous manner to our observations with ITX5061 [10]. This raises the question of the magnitude of HCV RNA decline that is possible following treatment with agents targeting the entry step of the viral lifecycle. The fate of viral particles following transplantation is challenging to study. Studies of HCV replication kinetics and quasispecies diversity post liver transplant suggest that 3-4% of circulating virus is produced from sites outside the liver [2-4]. Assuming these estimates are accurate, it would not be possible for an inhibitor targeting the viral entry process to depress the plasma viral burden by more than 96-97%. It may therefore be unrealistic to expect larger reductions in HCV RNA with even the most potent entry inhibitors. This is in contrast to the effects of DAAs that are increasingly being used in both patients waiting for liver transplantation and in patients post-transplantation where rapid reductions in plasma HCV RNA are observed [25]. *In vitro* studies and small animal models that support HCV replication show that entry inhibitors can prevent infection and suggest a role for these agents in the transplantation setting [15, 26]. Since these model systems lack extrahepatic sites for virus replication they are likely to overestimate the efficacy of entry inhibitors in patients undergoing liver transplantation as evidenced in our current study.

ITX5061 was recently reported to have a minimal effect on HCV RNA burden in subjects with chronic genotype 1 infection [27]. A number of explanations that are also relevant to the current study were proposed including insufficient dosing, duration of treatment or the presence of pre-existing drug resistant mutations. Zhu and colleagues reported that a single amino acid substitution in HCV E2 at residue N415D conferred resistance to ITX5061 *in vitro* [19]. However, inspection of our UDPS dataset from treated patients failed to identify a single occurrence of this polymorphism, demonstrating that mutations at this site did not explain the limited efficacy of ITX5061 in our study. An alternative explanation for the modest effects of ITX5061 on HCV replication may be insufficient drug targeting hepatocellular SR-BI. However, pharmacokinetic analysis demonstrated drug plasma levels in excess of the EC_{90} required to inhibit HCV entry *in vitro*. Since ITX5061 is cleared by the liver we anticipate that hepatic levels will exceed those measured in the plasma and so it is unlikely that insufficient drug contributed to the limited efficacy of *in vivo*. We failed to see any association between drug levels and the early rise in HCV RNA observed in two patients. Masson et al reported increased serum HDL as a biomarker for ITX5061 activity [17]. However, we did not identify any significant differences in serum HDL levels between the treated and untreated groups (**Suppl. Fig.3**). The marked decrease in HDL observed in both groups following transplantation is likely associated with the dyslipidaemia of patients with critical illness that is well recognised [28] and may explain the failure to demonstrate a difference between the groups in this study. Additional factors such as the IL-28B polymorphism may define innate interferon signalling in the allograft that may affect early stage HCV replication kinetics. Unpublished data show no significant role for IL28B genotype of the donor or recipient in early stage HCV replication kinetics (X. Forns and S. Perez-del-Pulgar, personal communication). We observed an apparent genotype dependent response to ITX5061 favouring genotype 1 infected patients. Whilst there is no *in vitro* evidence for genotype specific effects of ITX5061, these data support a model where genotype 3 viruses may be less dependent on SR-BI for entry into hepatocytes *in vivo*. This finding is analogous to early studies with the protease inhibitor telaprevir that showed *in vitro* activity against genotype 3 viruses but limited efficacy *in vivo* [29]. Given the relatively small number of patients studied, it is possible that our observations are made by chance, and that there is no genotype-dependent effect of ITX5061 on viral kinetics and further clinical studies are required to explore this further.

SR-BI is expressed on the endothelial cells lining the sinusoids that play a major role in the clearance of adenoviral particles from the circulation [30]. However, their role in HCV infection is debated and the scavenging activity of SR-BI may contribute to clearing HCV particles from the periphery [31, 32]. ITX5061 had no effect on HCV clearance rates suggesting a minimal role for endothelial SR-BI in this process. In contrast, ITX5061 had a significant reduction on HCV RNA levels 24h post transplant, suggesting a block to extracellular virus production from newly infected hepatocytes. This conclusion is supported by data showing an eclipse period of approximately 9h between virus entry into a hepatoma cell and the detection of extracellular progeny virus [33].

This trial provided the opportunity to study the impact of an entry inhibitor on HCV evolution in the first week after transplantation. We observed a complex and evolving pattern of HCV variants in the plasma of untreated patients. In contrast to earlier reports that suggest a ‘founder effect’ leads to

monotypic infection of the allograft [34-36], our data are consistent with a recent report demonstrating that viral diversity is maintained in the first months after liver transplant [37]. The apparent discrepancies between these studies most likely reflect the increased depth of ultra deep sequencing methodologies used to study the HCV infected allograft. Importantly, we saw limited evidence for viral genetic evolution in treated patients, demonstrating efficacy of ITX5061 *in vivo*.

The results of this study need to be viewed in the context of recent and ongoing developments in the field of therapy for HCV infection, including the recent introduction of DAAs targeting the viral polymerase, protease, and non-structural protein NS5A. Clinical trials of various DAAs demonstrate high cure rates (>90% in most studies) in selected patient populations. However, there is limited information on the use of DAAs in the liver transplantation setting, although data suggest that new DAA combinations are likely to be effective at curing infection after transplantation [25, 38, 39]. Future treatment strategies are likely to include DAAs to cure HCV infection before or after liver transplantation. HCV entry inhibitors, like ITX5061, may have a role in patients who are transplanted during antiviral treatment or in those who develop DAA resistance undergoing retreatment.

In conclusion, ITX5061 treatment was safe and had no significant drug-drug interactions. The drug reduced HCV RNA in the plasma, in particular in genotype 1 infected patients, and restricted HCV sequence evolution. The relatively modest reduction in HCV RNA levels argues for a role of extrahepatic sites of replication in infection [40] and suggest that future studies of entry inhibitors may be more effectively utilized in combination with replication inhibitors to eradicate HCV infection.

Table 1. Patient demographics. G, genotype; HCC, hepatocellular carcinoma; IQR, interquartile range; MELD, model for end-stage liver disease. Continuous variables compared with the [§]Mann-Witney test, and categorical variables compared using [+]Fisher's exact test.

Table 2. Patient safety information. All adverse events were recorded. The highest grade of each adverse event for all participants was categorised. Adverse events were graded using the National Cancer Institute Common Terminology Criteria v.4.0.

Table 3. Tacrolimus dosing information. Tacrolimus dose and trough concentrations were recorded at day 3, day 5, and day 7 after transplantation. All comparisons were made using the Mann-Witney test. IQR, interquartile range.

Fig.1. Recruitment flowchart for ITX5061 trial. Of 33 individuals who consented to participate 10 were not included in the final study. The first 13 patients who did not receive drug were sampled under the same regimen as the remainder to give comparison samples for the subsequent analyses. HCC, hepatocellular carcinoma.

Fig.2: Plasma HCV RNA kinetics in the first 24 hours after transplantation. The median (± interquartile range) viral load values for plasma samples taken over the first 24 hours post-transplant are shown relative to the sample taken at the end of the anhepatic phase (time zero, panel **A**). The HCV RNA values in all patients at 24 hours were compared using the Mann-Witney test (**B**).

Fig.3: HCV RNA in ITX5061 treated and untreated patients. The individual viral load values for plasma samples taken over the first week post-transplant are shown relative to the sample taken at the end of the anhepatic phase (time zero, panel **A**). The area over the curve (AOC) of HCV RNA values in the first transplant week (**B**) were compared using the Mann-Witney test. Weekly monitoring of HCV RNA levels continued in both untreated and ITX5061 treated patients after the first week post transplant with the boxed area indicating the treatment period (**C**). The AOC of values for changes in the first month after transplantation were compared using the Mann-Witney test (**D**).

Fig.4: HCV RNA in ITX5061 treated and untreated genotype 1 infected patients. The individual viral load values for plasma samples from genotype 1 infected patients are shown relative to the sample taken at the end of the anhepatic phase (time zero, panel **A**). The area over the curve (AOC) of HCV RNA values in the first transplant week were compared using the Mann-Witney test (**B**).

Fig.5: Effect of ITX5061 on HCV genetic diversity and genetic change during therapy. Ultra-deep pyrosequencing of HCV E1E2 variants in the plasma show two patterns of intra-sample diversity: high variability (>10%, upper heatmap) or a lower level of diversity (< 10%, lower heatmap). Each box in the heatmap corresponds to an amino acid position within E1E2, where diversity is quantified according to a colour gradient where black corresponds to no diversity, dark blue less than 10% diversity and brighter colours, e.g. green equate to higher diversity up to 50% (**A**). Average pairwise diversity between sequences in plasma samples from untreated (UT) and ITX5061 treated patients at 0 and 7 days were determined, no significant differences were seen between any of the four groups. Open symbols indicate samples derived from patients with fast replication kinetics, and closed

symbols with slow kinetics (**B**). Assessment of sequence change in the HVR region during the first week after transplant shows a significant reduction in the amount of change detected in ITX5061-treated patients (Mann-Witney p=0.027). Open symbols indicate samples derived from patients with fast replication kinetics, and closed symbols those with slow kinetics (**C**).

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	Untreated (n=13)	ITX5061 (n=10)	Comparison
Age, y			
Median (IQR)	57 (49 – 58)	56 (48 – 51)	0.87[§]
Male gender			
No. (%)	11 (85)	10 (100)	0.49[+]
Ethnicity			
No. Caucasian (%)	9 (69)	10 (100)	0.10[+]
Weight, kg			
Median (IQR)	88 (73 – 98)	93 (91 – 95)	0.73[§]
BMI, kg/m^2			
Median (IQR)	27.3 (26.7 – 31.1)	29.6 (26.7 – 31.5)	0.84[§]
Indication			
No. Liver failure (%)	8 (62)	8 (80)	
No. HCC (%)	5 (38)	2 (20)	0.41[+]
MELD			
Median (IQR)	15 (11 – 17)	15 (11 – 16)	0.99[§]
HCV RNA, $\log_{10}$ IU/mL			
Median (IQR)	5.8 (4.7 – 6.0)	5.3 (4.5 – 6.1)	0.68[§]
Genotype			
No. G1 (%)	7 (54)	6 (60)	
No. G2 (%)	1 (8)	0 (0)	
No. G3 (%)	4 (30)	4 (40)	
No. G4 (%)	1 (8)	0 (0)	1.00[+]
Prior antiviral therapy			
Yes (%)	10 (77)	9 (90)	0.60[+]
Donor age			
Median (IQR)	50 (45 – 56)	54 (42 – 60)	0.94[§]
Donor type			
Donation after brain death	9	6	
Donation after cardiac death	4	4	0.69[+]
Whole or split liver transplant			
Whole	11	9	
Split	2	1	1.00[+]
Cold ischaemia time, minutes			
Median (IQR)	534 (494 – 615)	490 (454 – 517)	0.07[§]
Warm ischaemia time, minutes			
Median (IQR)	41 (35 – 42)	38(37 – 44)	0.77[§]

Table 1. Patient demographics. BMI, body mass index**;** G, genotype ; HCC, hepatocellular carcinoma; IQR, interquartile range; MELD, model for end-stage liver disease. Continuous variables compared with the [§]Mann-Witney test, and categorical variables compared using [+]Fisher's exact test.

	Grade 1		Grade 2		Grade 3		Grade 4	
	Untreated (n=13)	ITX5061 (n=10)	Untreated (n=13)	ITX5061 (n=10)	Untreated (n=13)	ITX5061 (n=10)	Untreated (n=10)	ITX5061 (n=10)
Blood and lymphatic system disorders	4	5	8	4	9	11	1	1
Cardiac disorders	0	0	5	1	3	0	2	0
Gastrointestinal disorders	8	7	17	20	3	1	0	0
General disorders and administration site condition	14	4	5	3	1	0	0	0
Infections and infestations	1	0	3	2	0	0	2	0
Investigations	24	26	35	16	35	27	17	11
Metabolism and nutrition disorders	2	2	22	26	9	3	2	1
Musculoskeletal and connective tissue disorders	1	2	2	0	0	0	0	0
Nervous system disorders	7	8	4	2	0	0	1	0
Psychiatric disorders	5	2	6	4	2	1	2	0
Renal and urinary disorders	0	2	0	0	1	0	0	1
Respiratory, thoracic, and mediastinal disorders	2	2	2	3	5	1	0	0
Skin and subcutaneous tissue disorders	1	1	4	0	0	0	0	0
Vascular disorders	3	0	8	7	5	6	1	0

Table 2. Patient safety information. All adverse events were recorded. The highest grade of each adverse event for all participants was categorised. Adverse events were graded using the National Cancer Institute Common Terminology Criteria v.4.0.

		Untreated (n=13)	ITX5061 (n=10)	Comparison
Tacrolimus daily dose (mg)				
Median (IQR)	Day 3	8 (6 – 8)	6 (6 – 7)	0.21
	Day 5	8 (6 – 8)	6 (5 – 8)	0.17
	Day 7	6 (6 – 8)	6.5 (6 – 8)	0.89
Tacrolimus trough concentration (µg/L)				
Median (IQR)	Day 3	5.0 (3.8 – 6.4)	5.7 (3.1 – 8.1)	0.79
	Day 5	7.1 (5.6 – 8.0)	5.4 (4.6 – 7.6)	0.38
	Day 7	7.2 (5.8 – 8.8)	5.4 (5.0 – 6.3)	0.14

Table 3. Tacrolimus dosing information. Tacrolimus dose and trough concentrations were recorded at day 3, day 5, and day 7 after transplantation. All comparisons were made using the Mann-Witney test. IQR, interquartile range.

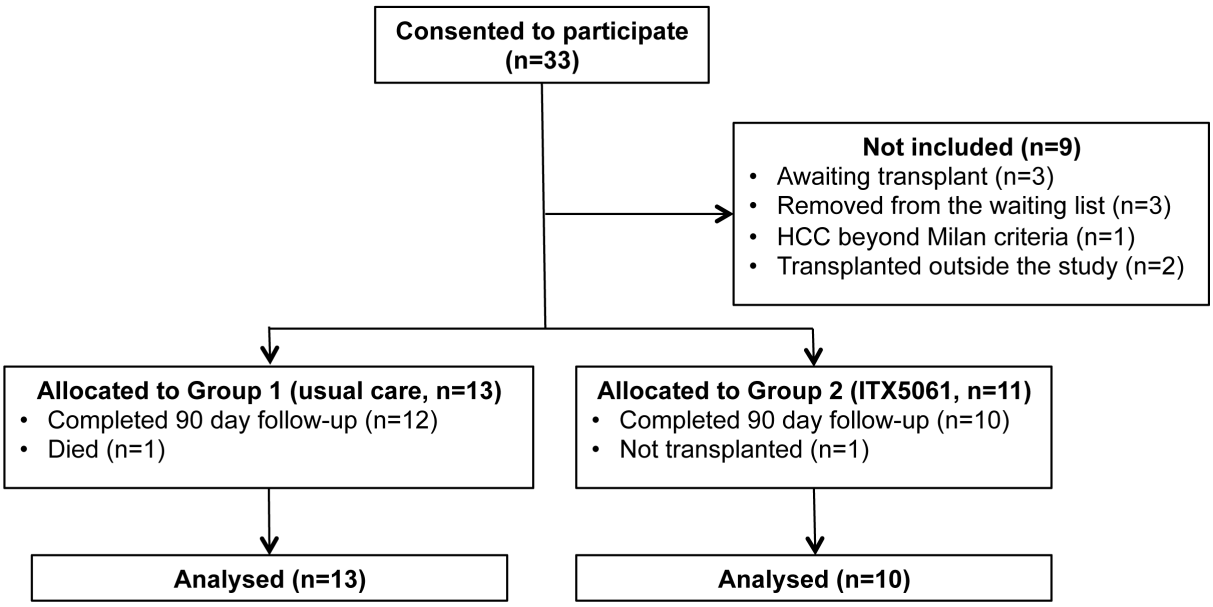

Fig.1. Recruitment flowchart for ITX5061 trial. Of 33 individuals who consented to participate 10 were not included in the final study. The first 13 patients who did not receive drug were sampled under the same regimen as the remainder to give comparison samples for the subsequent analyses. HCC, hepatocellular carcinoma.

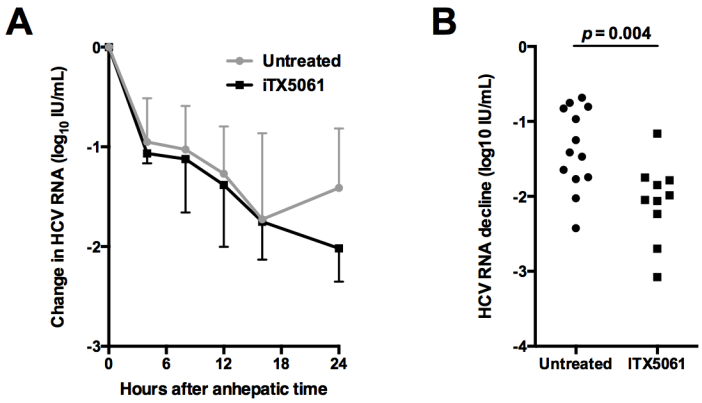

Fig.2: Plasma HCV RNA kinetics in the first 24 hours after transplantation. The median (± interquartile range) viral load values for plasma samples taken over the first 24 hours post-transplant are shown relative to the sample taken at the end of the anhepatic phase (time zero, panel **A**). The HCV RNA values in all patients at 24 hours were compared using the Mann-Witney test (**B**).

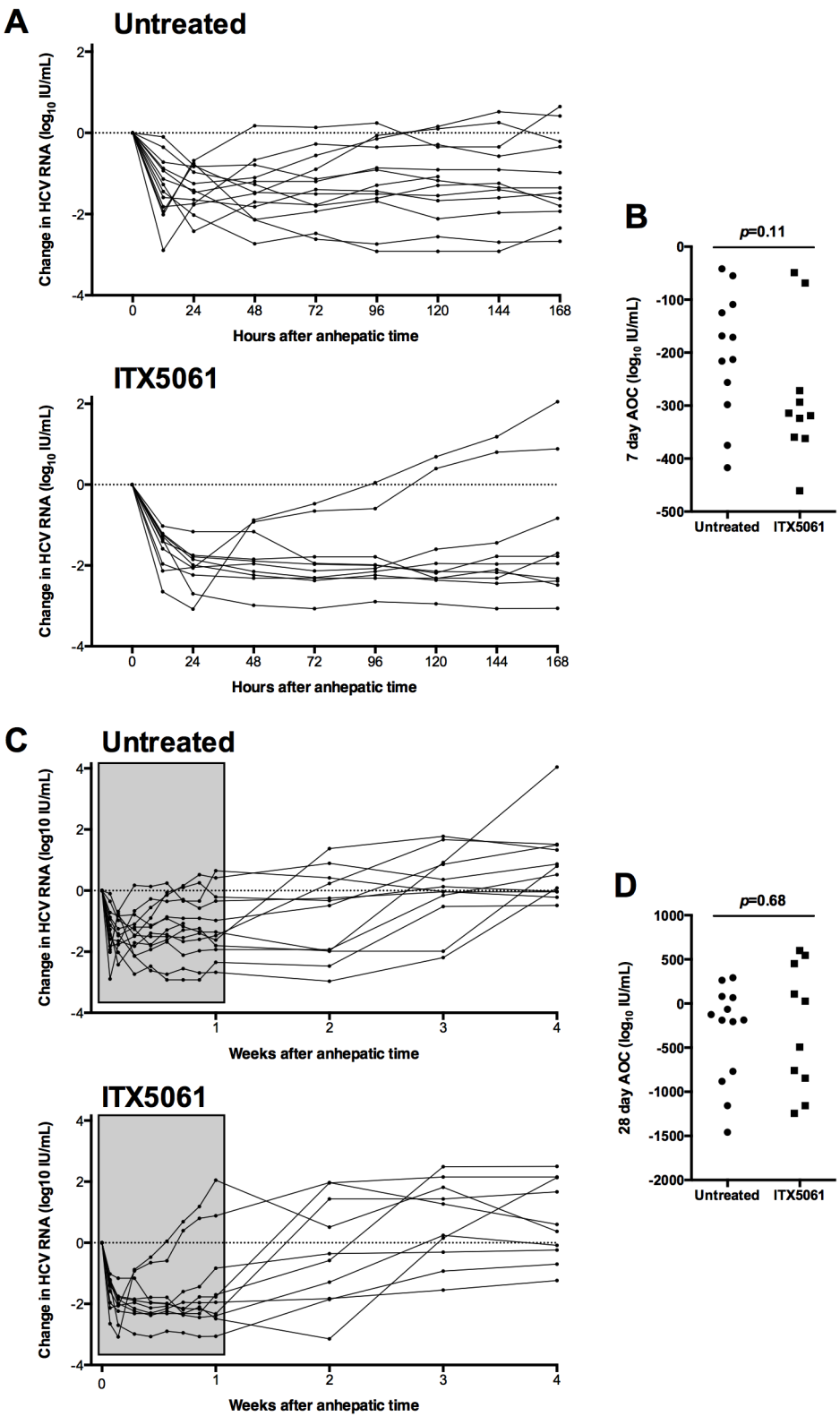

Fig.3: HCV RNA in ITX5061 treated and untreated patients. The individual viral load values for plasma samples taken over the first week post-transplant are shown relative to the sample taken at the end of the anhepatic phase (time zero, panel **A**). The area over the curve (AOC) of HCV RNA values in the first transplant week (**B**) were compared using the Mann-Witney test. Weekly monitoring of HCV RNA levels continued in both untreated and ITX5061 treated patients after the first week post transplant with the boxed area indicating the treatment period (**C**). The AOC of values for changes in the first month after transplantation were compared using the Mann-Witney test (**D**).

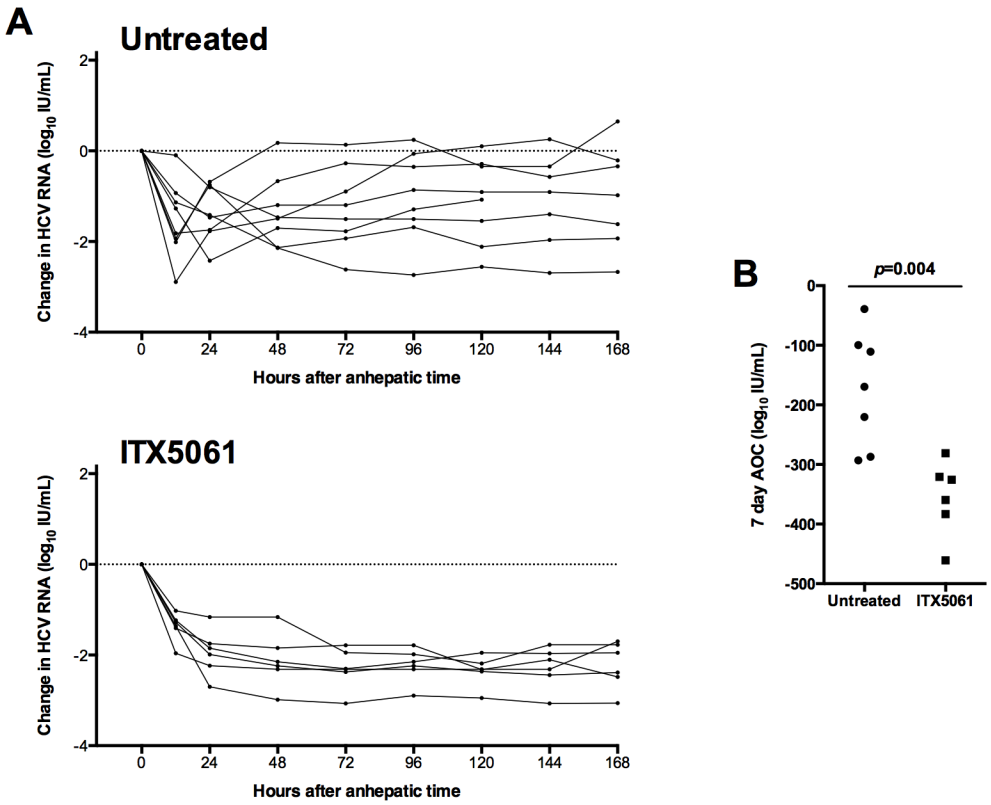

Fig.4: HCV RNA in ITX5061 treated and untreated genotype 1 infected patients. The individual viral load values for plasma samples from genotype 1 infected patients are shown relative to the sample taken at the end of the anhepatic phase (time zero, panel **A**). The area over the curve (AOC) of HCV RNA values in the first transplant week were compared using the Mann-Witney test (**B**).

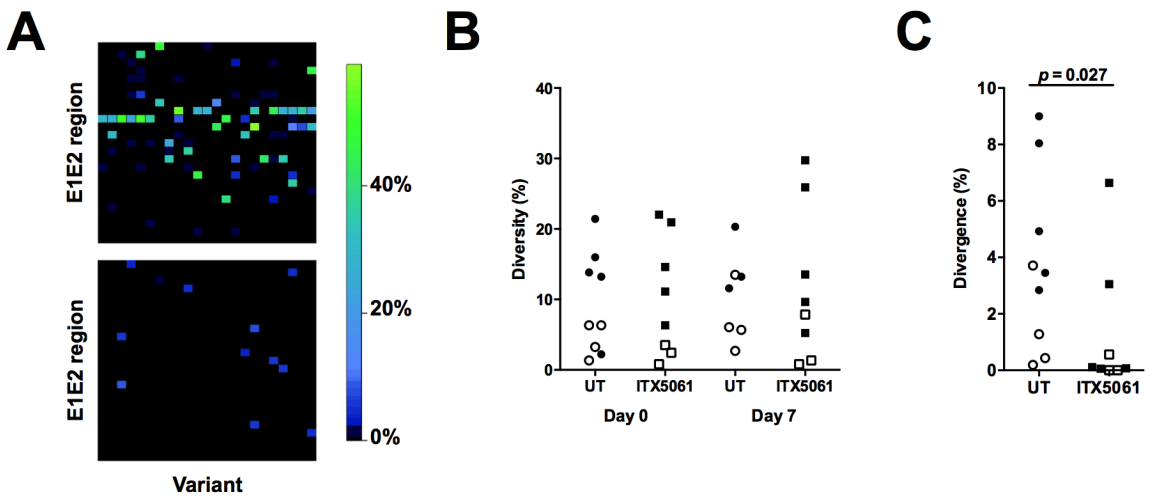

Fig.5: Effect of ITX5061 on HCV genetic diversity and genetic change during therapy. Ultra-deep pyrosequencing of HCV E1E2 variants in the plasma show two patterns of intra-sample diversity: high variability (>10%, upper heatmap) or a lower level of diversity (< 10%, lower heatmap). Each box in the heatmap corresponds to an amino acid position within E1E2, where diversity is quantified according to a colour gradient where black corresponds to no diversity, dark blue less than 10% diversity and brighter colours, e.g. green equate to higher diversity up to 50% (**A**). Average pairwise diversity between sequences in plasma samples from untreated (UT) and ITX5061 treated patients at 0 and 7 days were determined, no significant differences were seen between any of the four groups. Open symbols indicate samples derived from patients with fast replication kinetics, and closed symbols with slow kinetics (**B**). Assessment of sequence change in the HVR region during the first week after transplant shows a significant reduction in the amount of change detected in ITX5061-treated patients (Mann-Witney *p*=0.027). Open symbols indicate samples derived from patients with fast replication kinetics, and closed symbols those with slow kinetics (**C**).