

ORIGINAL ARTICLE

Torque Teno virus: any pathological role in liver transplanted patients?

Patrizia Burra,¹ Annalisa Masier,¹ Caterina Boldrin,² Arianna Calistri,² Elisabetta Andreoli,³ Marco Senzolo,¹ Manuel Zorzi,⁴ Dino Sgarabotto,⁵ Maria Guido,⁶ Umberto Cillo,⁷ Daniele Canova,¹ Mauro Bendinelli,³ Mauro Pistello,³ Fabrizio Maggi³ and Giorgio Palù²

1 Gastroenterology Section, Department of Surgical and Gastroenterological Sciences, University of Padova, Padova, Italy

2 Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Padova, Italy

3 Virology Section and Retrovirus Centre, Department of Experimental Pathology, University of Pisa, Pisa, Italy

4 Venetian Tumor Registry, Istituto Oncologico Veneto, Padova, Italy

5 Department of Infectious Diseases, University of Padova, Padova, Italy

6 Pathology Institute, University of Padova, Padova, Italy

7 Surgical Clinic, Department of Surgical and Gastroenterological Sciences, University of Padova, Padova, Italy

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Correspondence

Patrizia Burra MD, PhD, Gastroenterology, Department of Surgical and Gastroenterological Sciences, Padova University Hospital, Via Giustiniani 2, 35128 Padova, Italy. Tel.: +39 049 8212892; fax: +39 049 8760820; e-mail: burra@unipd.it

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Summary

Few studies have been performed on the prevalence of Torque Teno Virus (TTV) infection in liver transplant (LT) recipients. The aim of this study was to assess the prevalence, viremia and genogroup pattern of TTV among LT patients and to ascertain whether TTV causes liver damage in liver transplanted patients with biochemical and histological changes of unknown origin. Twenty-five patients were evaluated before and after LT; 80 healthy subjects were considered as controls. Serum samples were serially obtained from all the patients before LT and thereafter at 3, 6 and 12 months post-transplant. Serum TTV-DNA and genogroups were assessed by PCR. Patients underwent protocol serial liver biopsies at 6 and 12 months after LT. Results were compared using the Chi-squared tests, McNemar's and Student's *t*-tests. TTV-DNA was found in 25/25 patients before LT and in 60/80 blood donors ($P < 0.01$). The TTV-DNA load increased significantly after LT ($P < 0.001$). TTV-DNA was significantly higher in patients on calcineurin inhibitors (CNI) and azathioprine or mycophenolate mofetil than in patients on CNI alone ($P = 0.04$) at 3 months after LT. Genogroup analysis showed a significant increase in genogroup 5 positivity after LT. No differences were seen in the viremia of patients compared according to their viral versus other etiologies of their liver disease before transplantation. Viremia and TTV genotype patterns did not correlate with the presence of hypertransaminasemia or histological liver damage of unknown etiology. The prevalence of TTV-DNA was significantly higher in patients with liver cirrhosis than in controls and the viral load was significantly higher after LT than beforehand. On the basis of our data, TTV does not seem to cause liver damage following LT, although larger studies with a long-term follow up are needed to confirm these findings.

Introduction

The Torque Teno virus (TTV) is a nonenveloped, single-stranded, circular DNA virus that was first identified in

the serum of a patient with post-transfusion hepatitis of unknown etiology [1]. Its genomic organization has led TTV to be classified provisionally in the 'floating' genus *Anellovirus*. The TTV genome is organized into a poten-

tially coding region of ~2.6 kb, including two major open reading frames (ORFs), ORF1 and ORF2 [2], and an untranslated region (UTR) of ~1.2 kb. TTV exhibits a marked genetic diversity, which can easily be analyzed by sequencing a region encompassing 222 nucleotides of the ORF1 N22 segment [3]. TTV transmission is mainly parenteral, but the virus can also be isolated in other biological specimens, such as respiratory secretions, feces, saliva, tears and semen.

The specific pathogenic role of TTV is still not known. Given its involvement in a few cases of non A–E hepatitis [4] and its isolation in cases of hepatitis of unknown etiology, a role for TTV has been suggested in the pathogenesis of liver disease, but available data are still controversial. As the prevalence of TTV in the general population is as high as 90% [5], its contribution to liver disease would demand specific conditions resulting from a combination of different host, viral and environmental factors, although the factors potentially capable of contributing to the clinical course of the infection are yet to be characterized.

We reported that nearly 30% of biopsies from liver-transplanted patients show minimal histological changes (classifiable as ‘not otherwise specified’) in cases in which all risk factors – including HBV, HCV, other hepatotropic viruses, rejection and drug toxicity – can be ruled out and no other causes of these anomalies can be identified [6]. We also found, during a 6- to 96-month follow up, that 25% of the patients transplanted for HBV-related cirrhosis had histologically demonstrable chronic hepatitis despite persistent HBsAg negativity and no HBV-DNA; here again, none of the common risk factors (e.g. alcohol and drug abuse) were identified in these patients [7]. Both series of patients revealed liver anomalies of unknown etiology, which meant that the risk and the rate of any progression of the patient’s liver disease was unpredictable, with a negative fallout on patient management.

The aim of this study was to evaluate the prevalence, viremia and genogroup pattern of TTV among LT patients and to ascertain whether TTV is responsible for liver damage in liver transplanted patients with biochemical and histological changes of unknown origin, or whether it exacerbates the damage caused by other viruses.

Materials and methods

Study population

The study population consisted of 25 patients (20 males and five females, mean age 50.26 years, range 24–65 years) randomly recruited from among patients listed for liver transplantation at the Gastroenterology Section,

Department of Surgical and Gastroenterological Sciences of Padova University Hospital, Italy, over a period of 5 years (1996–2001), who consented for blood sampling to be carried out at the time of their transplantation and at 3, 6 and 12 months post-transplantation, and also for liver biopsies to be performed at 6 and 12 months after liver transplantation.

Eighty healthy blood donors were considered as controls.

Liver function tests [aspartate aminotransferase (IU/l), alanine aminotransferase (IU/l), gamma glutamyl transpeptidase (IU/l), alkaline phosphatase (IU/l), and total bilirubin (mg/dl)], serum levels of immunosuppressants and TTV in the plasma were analyzed in all patients at the same time points.

Before liver transplantation, anti-HCV antibodies (EIA II-III or RIBA III) were assessed by qualitative and quantitative HCV-RNA (RT-PCR, Amplicor) in all HCV-positive patients, and HBV-DNA (PCR Monitor Amplicor) and hepatitis D virus (HDV) markers (total anti-HDV IgG, ELISA II) were assayed in all HBsAg-positive patients.

After liver transplantation, HBsAg, HBsAb (ELISA II) and HBV-DNA were assessed in HBV patients who had been given immunoprophylaxis indefinitely to maintain anti-HBs titers ≥ 250 IU/l, plus lamivudine 100 mg/day. HBIG therapy was discontinued in patients who became HBsAg-positive after liver transplantation [8].

Quantitative and qualitative HCV-RNA was performed at the same time in all anti-HCV positive patients; antiviral therapy with pegylated interferon and ribavirin for 6–12 months was given for fibrosis stage 2 (according to Scheuer [9]) or higher. Treatment was started after cardiac, renal and neurological assessment, and after excluding any ongoing episodes of acute or chronic rejection, as reported elsewhere [10,11].

A post-transplant psychosocial assessment was conducted in patients transplanted for alcoholic liver cirrhosis, using a semi-structured interview administered by a physician specializing in alcohol-related problems. Alcohol recidivism was defined according to Lucey as any alcohol consumption after liver transplantation [12].

TTV detection, quantification and genotyping

TTV-DNA was extracted from 200 μ l of plasma using the standard proteinase K phenol–chloroform method. Plasma specimens were tested for TTV with a single-step amplification assay, using primer set mapping at UTR level, as reported by Okamoto *et al.* [13] TTV-DNA was quantified by TaqMan real-time PCR, specific for highly conserved segments of viral UTR, as described elsewhere

[14]. All samples from individual patients were assayed simultaneously in triplicate.

TTV-positive DNA extracts were genotyped by running 5 separate genogroup-specific PCRs, using five primer sets, as reported in the literature [15]. Amplification products from the group-specific PCR assays were sequenced directly using the Big Dye Terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA). Cycles were sequenced in both directions using an automatic DNA sequencer (ABI model 3100; PE Biosystems, Foster City, CA, USA). Nucleotide sequences were aligned with database reference sequences.

Liver histology

Protocol liver biopsies were obtained at 6 and 12 months after liver transplantation using a 16–18 gauge needle; the tissue sample was fixed in formalin and embedded in paraffin, then stained with hematoxylin and eosin. Recurrent HCV and/or HBV infection was evaluated according to Scheuer [9] and relapses into alcoholic liver disease according to the Lancet Group IS features [16], while nonspecific mild histological changes were reported as not otherwise classified, according to Pappo's terminology [17]. Acute and chronic rejections were assessed according to the Banff classification [18]. A semi quantitative score was used to classify liver damage after transplantation as: no histological damage; nonspecific damage, mild chronic hepatitis (fibrosis stage 1); moderate chronic hepatitis (fibrosis stage 2); severe chronic hepatitis (fibrosis stage 3).

Statistical evaluation

Data are given as mean \pm standard errors. Data were compared using the Chi-squared test, McNemar's test for paired data and Student's *t*-test.

Results

During the study period (between 1996 and 2001), 166 transplants were performed in 155 patients; the 5-year patient- and graft survival rates were 84% and 82%, respectively.

The reason for liver transplantation was HBV/HDV or HCV cirrhosis (conventionally termed as 'viral' in this paper) in 14 patients (2 HBV, 1 HBV/HDV, 8 HCV, 1 HBV/HDV/HCV, 2 HBV/HCV), and non HBV/HDV/HCV ('nonviral') cirrhosis in 11 (seven alcoholic, one cryptogenic, one polycystic, one amyloid, one congenital liver fibrosis).

Immunosuppression consisted of 500 mg of intravenous methylprednisolone given intraoperatively and then

125 mg on day 1 and 40 mg on day 2, then oral prednisone 25 mg/day up to day 30, 15 mg/day in the second month and 5 mg/day in the third. One patient only received induction with basiliximab at the time of the transplant, without any steroids. The immunosuppressants were mainly calcineurin inhibitors. Up until the year 2000, cyclosporine A C0 levels were maintained at 250–300 ng/ml for the first 3 months, then at 150–250 ng/ml up to 6 months, at 150–200 ng/ml up to 12 months and at 100–150 ng/ml up to 36 months after LT. After the year 2000, cyclosporine A C2 levels were maintained at 800–1200 ng/ml for the first 3 months, then at 600–800 ng/ml up to 6 months, at 400–600 ng/ml up to 12 months and at 300–500 ng/ml up to 36 months after liver transplantation. Tacrolimus trough levels were kept at 10–15 ng/ml for the first 6 months, then at 8–12 ng/ml up to 12 months, at 6–10 ng/ml up to 24 months and at 5–8 ng/ml up to 36 months after LT.

Azathioprine (1.5 mg/kg) up until the year 2000 and mycophenolate mofetil (1.5–2 g) thereafter were added when the patient's serum creatinine was higher than 200 μ mol/l in order to contain the cyclosporine or tacrolimus dosage.

Maintenance immunosuppression as of 3 months after LT was as follows: 19 patients received calcineurin inhibitors (CNI) alone (nine on tacrolimus, 10 on cyclosporine A), and six patients were given CNI in association with a second agent (tacrolimus and azathioprine in 1, tacrolimus and rapamycin in 1, cyclosporine A and azathioprine in 1, cyclosporine A and mycophenolate mofetil in 3).

After LT, the biopsy-proven acute rejection rates were 8.3%, 1.7%, 2.8% and 1.6% at 6, 12, 24, and 36 months, respectively. The rate of biopsy-proven chronic rejection was 2.8% after 12 months and 1.6% after 24 months.

All patients were given Herpesvirus prophylaxis with oral acyclovir at a dose of 1000 mg/day for the first 3 months after LT.

One of the six patients with HBV cirrhosis became HBsAg positive and HBV-DNA positive 12 months after liver transplantation: this patient was treated with lamivudine and developed lamivudine resistance, so adefovir dipivoxil was added and the HBV-DNA became negative within 6 months.

Eight of 11 patients (72.7%) transplanted for HCV-related cirrhosis had histologically proven recurrent HCV hepatitis, 3 months (three patients), 6 months (three patients) and 12 months (two patients) after LT. Three of the eight patients with recurrent HCV were given 180 μ g/week of pegylated interferon and 800 mg/day of ribavirin: 2/3 patients completed the 12-month course of treatment and their HCV-RNA assay was negative another 6 months later; the third patient failed to respond so the treatment was stopped after 6 months.

None of the nine patients transplanted for alcoholic cirrhosis relapsed into alcohol consumption judging from their blood samples, psychosocial assessment, liver function tests, validated questionnaires and liver histology.

TTV evaluation

Before LT, 25/25 cirrhotic patients (100%) were positive for TTV, as opposed to 60/80 healthy individuals (75%) (Chi-squared test: $P < 0.004$).

Before surgery, the TTV-DNA load was similar in the 25 patients (4.2 ± 0.6 log copies/ml) and in the 60 healthy controls (4.6 ± 1.0 log copies/ml), but it became significantly higher in the transplanted patients at 3 months (6.8 ± 0.4 log copies/ml), 6 months (7.0 ± 0.5 log copies/ml) and 12 months (6.4 ± 0.6 log copies/ml) after LT ($P < 0.001$). No differences in viremia were seen at 6 and 12 months after LT. When patients were divided into viral versus nonviral cases, the TTV-viral load was similar in the two groups at the baseline and also at 3, 6 and 12 months after LT (Fig. 1), but when patients were grouped according to the type of immunosuppression received after LT, TTV-DNA was significantly higher in patients on calcineurin inhibitors and azathioprine or mycophenolate mofetil than in patients on CNI alone (7.35 ± 0.6 vs. 6.5 ± 0.38 log copies/ml; $P = 0.04$) 3 months after LT (Table 1). No differences emerged at 3, 6 and 12 months after LT between the groups of patients on tacrolimus versus cyclosporine therapy (data not shown); nor were there any differences in viremia between patients with normal liver function test results and those with hypertransaminasemia, or between patients with a normal liver histology and those with any degree of liver damage.

Table 1. TTV viral load according to amount of immunosuppression after liver transplantation over time.

Time after liver transplantation	Viremia Log copies/ml \pm ES		P-value
	CNI monotherapy	CNI+ Aza or MMF	
3rd month	6.5 ± 0.4	7.35 ± 0.6	0.045
6th month	6.5 ± 1	7.2 ± 0.9	0.25
12th month	6.2 ± 0.7	6.5 ± 1	0.62

There was a drop in TTV viremia and in the number of TTV genogroups during the follow up in the three out of 11 patients given antiviral therapy for recurrent HCV.

The composition and variation of the genogroups in patients and controls is shown in Table 2. There was no genogroup G2 in the healthy controls and the prevalence of G5 was lower than that in the patients, before or after LT ($P < 0.05$.) Genogroups G2 and G5 were poorly represented in the patients before LT, but the presence of G5 increased significantly afterwards ($P = 0.001$).

The pattern of the different genogroups changed over the 12 months after LT. At the baseline, four viral and two nonviral patients were infected with one genogroup, three nonviral patients were infected with two, and 16 viral and nonviral patients had three or more genogroups. A general trend towards an increasing number of different genogroups was seen in both viral and non-viral patients.

In the nonviral patient group, the proportion of patients positive for genogroups G1, G4 and G5 increased significantly over the 12-month follow up ($P < 0.05$), while in the viral group, only the proportion of patients positive for G2 and G5 rose significantly ($P < 0.05$). No

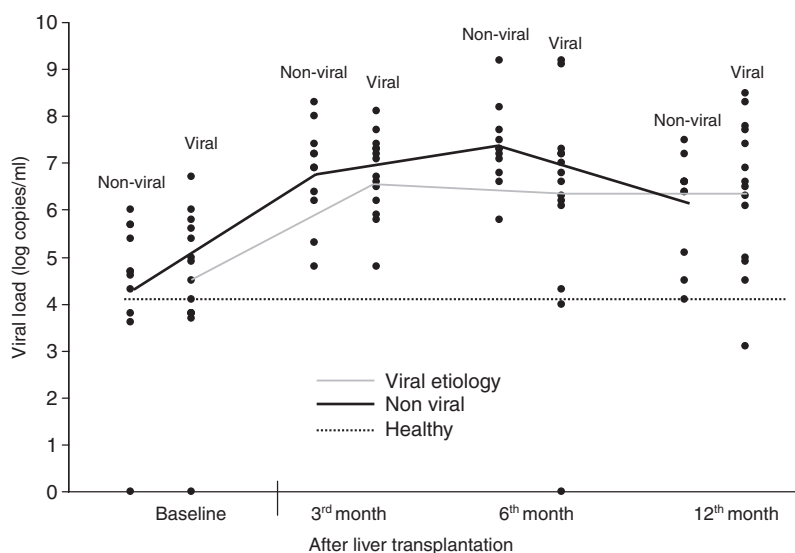


Figure 1 Longitudinal assessment of TTV viremia at the baseline and different time points after liver transplantation grouped according to viral and nonviral etiologies of liver disease. The dots represent single measurement of viremia and the line represents the trend of mean TTV viral load at baseline and different time points after liver transplantation.

Table 2. Distribution of TTV genogroups in healthy controls and in patients before and after liver transplantation.

Genogroup	Healthy controls (n = 30/80)	Patients (n = 25/25)				P-value
		Baseline	3rd month	6th month	12th month	
G1	67% (20/30)	72% (18/25)	92% (23/25)	88% (22/25)	88% (22/25)	n.s.
G2	0%*	40% (10/25)*	76% (19/25)	56% (14/25)	64% (16/25)	P = 0.0001*
G3	63% (19/30)	88% (22/25)	100% (25/25)	96% (24/25)	88% (22/25)	n.s.
G4	27% (8/30)	68% (17/25)	88% (22/25)	84% (21/25)	84% (21/25)	n.s.
G5	7%† (2/30)	28%‡,§ (7/25)	84% (21/25)	68% (17/25)	72%‡ (18/25)	P = 0.04† P = 0.0010‡

The statistical analysis between healthy controls and baseline was performed by χ^2 test; the statistical analysis between baseline and at 3, 6 and 12 months after liver transplantation was performed by McNemar's test for paired data. *P = 0.0001; †P = 0.04; ‡P = 0.0010.

correlation emerged between the genogroups and either histological liver damage or hypertransaminasemia.

Genogroup positivity, serum transaminases and histological damage are summarized in Table 3.

Episodes of hypertransaminasemia after liver transplantation

Thirteen of 25 patients experienced episodes of hypertransaminasemia after liver transplantation (Table 3).

Ten of these 13 patients had been transplanted for viral cirrhosis. One of these 10 patients suffered acute cellular rejection, while in 8/10 the liver damage was attributed to recurrent viral disease (7/8 HCV and 1/8 HBV). In the remaining patient, the reason for the hypertransaminasemia was not established (HBV-DNA, HCV-RNA and markers for EBV, CMV, HSV, VZV, HHV-6 and HHV-8 were negative).

In the 3/13 nonviral patients with hypertransaminasemia after LT, the liver function tests identified acute cel-

Table 3. ALT serum levels, histological damage and TTV genogroup expression at 6 and 12 months after liver transplantation in all patients enrolled in the study.

Patient	Etiology of liver disease	ALT at 6/12 months	Histological damage at 6/12 months	G1 at 6/12 months	G2 at 6/12 months	G3 at 6/12 months	G4 at 6/12 months	G5 at 6/12 months
1	HBV/HDV	N/>4x	Mild/moderate CH	+/+	+/+	+/+	+/+	+/-
2	HBV	2x < y < 4x	Rejection/rejection	+/+	-/+	+/+	+/+	+/+
3	HBV	<2x	Mild/mild CH	+/+	-/-	+/+	+/+	+/+
4	HBV/HDV/HCV	Norm.	Moderate/mild CH	-/-	-/-	+/-	-/-	+/+
5	HBV/HCV/Alcohol	2x < y < 4x	Nonspecific/nonspecific	+	+	+	-	-
6	HBV/HCV	>4/ND	Nonspecific/none	+/+	+/+	+/+	+/+	-/+
7	HCV	N/<2x	Mild/moderate CH	+/-	-/-	+/+	+/-	+/-
8	HCV	N/<2x	Mild/mild CH	-/+	-/+	+/+	-/+	-/+
9	HCV/Alcohol	Norm.	Mild/moderate CH	+/+	-/+	+/+	+/+	+/+
10	HCV	<2/>4x	Moderate/severe CH	+/+	+/+	+/-	+/+	+/+
11	HCV/Alcohol	<2/Norm.	Mild/mild CH	+/+	-/+	+/+	-/-	-/+
12	HCV	<2/Norm.	Moderate/moderate CH	+/+	+/+	+/+	+/+	+/+
13	HCV	Norm.	None/mild CH	+/+	+/+	+/+	+/+	+/+
14	HCV/Alcohol	Norm.	Nonspecific/nonspecific	-/+	-/-	+/+	+/+	-/+
15	Alcohol	Norm.	Moderate/mild CH	+/+	+/+	+/+	+/+	+/+
16	Alcohol	Norm.	Mild/mild CH	+/+	+/+	+/+	+/+	-/-
17	Alcohol	>4/<2x	Rejection/nonspecific	+/+	-/-	+/+	+/+	+/+
18	Alcohol	Norm.	Nonspecific/none	+/+	+/+	+/+	+/+	-/+
19	Alcohol	Norm.	Rejection/mild CH	+/+	+/-	+/+	+/+	+/+
20	Polycystic liver disease	Norm.	None/mild CH	+/+	-/-	-/+	-/+	-/-
21	Amyloidosis	Norm.	Nonspecific/none	+/+	-/-	+/-	+/+	+/-
22	Hepatic fibrosis	Norm.	Not done	+/+	-/+	+/+	-/+	+/+
23	Alcohol	Norm.	Mild/none	+/+	-/+	+/+	-/+	+/+
24	Cryptogenic	2x < y < 4x	Nonspecific/nonspecific	+/+	+/-	+/+	+/+	+/+
25	Alcohol	N/<2x	Mild/mild CH	+/+	-/+	+/+	-/+	+/+

ALT serum levels reported as fraction of the upper normal limit (n.v. 10–45 U/ml). Variability of viral load reported as percentage of variability between baseline and 6th month, and between baseline and 12th month values after liver transplantation. ALT, Alanine aminotransferase; CH, chronic hepatitis.

lular rejection in one patient, *de novo* HBV infection in one, and no known etiology in one, who was negative for HBV-DNA, HCV-RNA and for EBV, CMV, HSV, VZV, HHV-6 and HHV-8 markers.

In two patients with hypertransaminasemia of unknown etiology the viral load and TTV genogroup were much the same as in the patients with no hypertransaminasemia.

Histological damage

Twenty-four of the 25 patients had liver biopsies at 6 and 12 months after LT (one patient was at high risk of bleeding because of a low platelet count, so no liver biopsy was performed). The histological findings are shown in Table 3. Eleven out of 25 patients had at least one episode of acute cellular rejection, treated with steroid boluses in 9/11 cases and by stepping up the dose of calcineurin inhibitors in 2/11.

The only HBV-DNA positive patient had histologically proven recurrent HBV.

Among the 11 patients with nonviral liver disease, six out of the 10 liver biopsies taken at 6 or 12 months after LT provided histological proof of liver damage compatible with mild-to-moderate chronic hepatitis, i.e. mild chronic hepatitis with CMV infection in 1/6, mild chronic hepatitis of unknown cause in 4/6, and moderate chronic hepatitis of unknown cause in 1/6.

Discussion

This is the third study analysing the prevalence and possible pathogenic effect of TTV in liver transplanted patients [19,20] and is the first to quantify TTV viremia and defines genogroup longitudinally in liver transplanted patients before LT and at 3, 6 and 12 months after LT.

An epidemiological survey on the prevalence of TTV suggested that this infection is globally distributed [4], but there is no consensus in the literature as to its exact prevalence in healthy populations, probably because of the different specificity and/or sensitivity of the PCR-based methods used in different studies [21–24]. Primers using the ORF1 region are much more sensitive than primers using the UTR region, as shown by Genovese *et al.* [20], so we used both UTR and ORF1 primers to measure TTV prevalence in healthy individuals; this amounted to about 75%, a figure consistent with other studies on the basis of detection protocols characterized by a similar sensitivity and specificity [13,23,24].

We found a significantly higher prevalence of TTV in cirrhotic patients, although the viremia in cirrhotics and healthy populations was surprisingly similar. It is assumed that TTV must be very contagious, but how it spreads is

not very clear. Most screening procedures have found the highest rates of infection among patients who have had multiple transfusions [2], but the reported TTV viremia rates in the general population are too high to be justified by a blood-borne transmission alone. Several authors have demonstrated, in fact, that the oral-fecal route is the most likely route of transmission [25,26], and this may explain why cirrhotics, being frequently hospitalized, would have a higher prevalence of TTV infection than healthy controls.

As expected, TTV viremia increased significantly after liver transplantation, as reported in patients after kidney transplantation [27], and probably because of immunosuppressive therapy. The role of immunosuppression in TTV load was confirmed in this study by the significant difference recorded between patients on single versus multiple immunosuppressive drugs (7.35 ± 0.6 vs. 6.5 ± 0.38 log copies/ml; $P = 0.04$), though no differences emerged in relation to the type of calcineurin inhibitor used.

As reported by Shang *et al.* [19], we also found a higher prevalence of the G2 genogroup in patients, before and after LT, than in healthy controls, but there was also a higher prevalence of G5 in our study population, which increased significantly after LT, suggesting a specific sensitivity to immunosuppression.

Co-infection with TTV is common in patients with chronic HBV or HCV infection, but TTV does not seem to be pathogenic and no direct clinical association between TTV and liver disease has been clearly demonstrated [28]. We likewise found no difference in TTV viremia between viral and nonviral patient groups, nor did the TTV load seem to be influenced by co-infection with other viruses. As expected as a result of the antiviral activity of interferon (but never previously reported after LT), three patients in our study were given pegylated interferon and ribavirin and their TTV viremia dropped to nil. A temporary drop in TTV viremia had already been observed in patients with hepatitis C virus treated with interferon [28].

The pathogenic potential of TTV is still under debate. Attempts to link the virus to specific diseases have either been unsuccessful or still require to be confirmed, leaving the virus 'unattached' to any specific clinical disease [29,30]. In the liver transplantation setting, it is also particularly difficult to attribute a pathogenic role to TTV because various viral and host factors are involved. Although we focused on histological damage of unknown origin after liver transplantation, we could find no association with TTV. In the two patients with hypertransaminasemia and the three with histologically proven hepatitis of unknown etiology, inflammation correlated with neither TTV viral load nor genogroups, a finding consistent

with a previous study [14]. Horvath *et al.* [31] likewise found no association between TTV infection and postoperative complications in patients transplanted for liver cirrhosis of undefined etiology, despite a high prevalence of TTV; and Shang *et al.* [19] demonstrated no co-variation between TTV viral load and transaminase levels in hepatitis of unexplained origin after LT.

In conclusion, this prospective study found a higher prevalence of TTV in cirrhotic patients than in healthy controls, and this TTV load rises after liver transplantation depending on the amount of immunosuppression administered. The pathogenic role of TTV after liver transplantation remains a mystery, however, and studies are needed on larger series of patients with a long-term follow up.

Authorship

PB, AM, MS: designed the study and wrote the paper. AM: collected the data. CB, EA: performed research. MZ: analyzed the data. AC, DS, MG, UC, DC, MB, MP, FM, GP: contributed important reagents.

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