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Diagnosis of occult hepatitis C without the need for a liver biopsy

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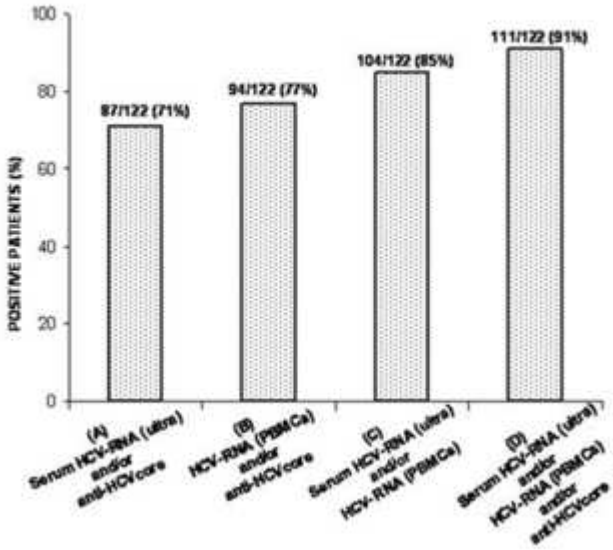

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Review

Diagnosis of occult hepatitis C without the need for a liver biopsy

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ABSTRACT

The diagnosis of occult hepatitis C virus (HCV) infection is based on the presence of HCV-RNA in the liver. This study aimed to evaluate the use of combining non-invasive assays to diagnose occult HCV. A total of 122 patients with occult HCV (HCV-RNA in the liver without detectable anti-HCV and serum HCV-RNA) and 45 patients with cryptogenic chronic hepatitis (without HCV-RNA in the liver and negative for anti-HCV and serum HCV-RNA) were included. HCV-RNA was tested in peripheral blood mononuclear cells (PBMCs) and in 2 mL of ultracentrifuged serum. Anti-core HCV was examined by a non-commercial enzyme-linked immunosorbent assay. All controls were negative for the three HCV markers studied. Among patients with occult HCV, 36% were anti-core HCV positive, 57% had serum HCV-RNA after ultracentrifugation and 61% had HCV-RNA in PBMCs. Combining the results of the assays, 91% of the patients were positive for at least one marker. Intrahepatic HCV-RNA load was significantly higher in patients who were positive simultaneously for the three HCV markers than in patients who were negative for all markers (p=0.006) and than in those with one or two HCV markers (p=0.039). Replication of HCV in liver was detected more frequently in patients with three (93%, p=0.002), two (82%, p=0.001) and one HCV marker (73%, p=0.011) than in those without markers (27%). In conclusion, testing for all these markers allows diagnosis of occult HCV without the need for a liver biopsy and these assays may help to elucidate the clinical significance of occult HCV infection.

Key words: Occult HCV; PBMCs; HCV-RNA; Anti-core HCV

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INTRODUCTION

Occult hepatitis C virus (HCV) infection was described in patients with chronic liver disease of unknown etiology and it is characterized by the presence of HCV-RNA in the liver despite persistent negative results for anti-HCV and for serum HCV-RNA, as tested by conventional assays [Castillo et al., 2004; Esaki et al., 2004; Comar et al., 2006; Pham et al., 2010]. In a high proportion of these patients, the antigenomic HCV-RNA strand (an ongoing HCV replication), is also detectable in hepatocytes. In addition, up to 70% of patients with occult HCV may have viral RNA in their peripheral blood mononuclear cells (PBMCs) [Castillo et al., 2004]. Although the detection of HCV-RNA in PBMCs allows diagnosis of occult HCV infection in certain cases [Carreño et al., 2004; Barril et al., 2008; De Marco et al., 2009; Zaghloul and El-Sherbiny, 2010], testing for viral RNA in the liver is the gold standard method to identify this occult infection. However, as a liver biopsy is not available in many cases, other diagnosis approaches have been investigated. In this regard, it was proven that HCV-RNA can be detected in the serum of patients with occult HCV after concentrating circulating viral particles by ultracentrifugation [Bartolomé et al., 2007]. A recent published work describes the development of an enzyme-linked immunosorbent assay (ELISA) that allows detection of IgG antibodies to an HCVcore-derived peptide in 31% of patients with occult HCV [Quiroga et al., 2009]. These tests provide new tools for the diagnosis of occult HCV [Fabrizi and Martin, 2008; Michalak and Pham, 2009], but the single use of one assay does not identify all patients with occult hepatitis C.

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The aim of this work was to study whether combining HCV-RNA detection in PBMCs and in ultracentrifuged serum, along with detection of anti-core HCV could avoid the need for a liver biopsy for the diagnosis of occult HCV infection.

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PATIENTS AND METHODS

Since the initial publication in 2004 describing the existence of an occult HCV infection in 57 anti-HCV and serum HCV-RNA negative patients [Castillo et al., 2004], 204 additional patients have been diagnosed at our Institution with occult HCV infection because they were found to be positive for HCV-RNA in the liver in the absence of serological HCV markers. Of these, 122 patients with available stored serum (at -80ºC) and PBMCs (at -20ºC in RNAlater solution, Ambion Inc., Austin, TX, USA), gave their consent to be included in this study. Table 1 shows the characteristics of these patients. Stored serum and PBMCs of 45 patients with non-viral chronic liver disease and who were negative for viral RNA in the liver, were included as a negative control group. The study was approved by the Local Ethical Committee and performed following the principles of the Declaration of Helsinki.

Isolation of total RNA

To confirm that all patients were serum HCV-RNA negative, total RNA was isolated from 250 µL of serum with Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA). After precipitation, the RNA pellet was dissolved in 10 µL of diethyl-pyrocarbonate (DEPC) treated water. Another 2 mL of the serum sample were ultracentrifuged over a 10% sucrose cushion for 17 h at 100,000 *g* and 4ºC to concentrate HCV particles [Bartolomé et al., 2007]. The pellet was dissolved in 250 µL of TE buffer (Tris-HCl 10 mmol/L, EDTA 10 mmol/L; pH 7.5), total RNA was isolated with Trizol LS Reagent (Invitrogen) and after precipitation, the pellet was dissolved in 10 µL of DEPC-treated water.

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Total RNA from PBMCs and liver biopsies was isolated with the SV Total RNA Isolation System (Promega Co., Madison, WI, USA). After precipitation, pellets were dissolved in DEPC-treated water and RNA concentration was determined by spectrophotometry.

HCV-RNA detection

The detection and quantitation of the 5' non-coding region of the genomic and antigenomic HCV-RNA strands was done by a strand-specific real-time RT-PCR, using the thermostable enzyme Tth for the synthesis of cDNA at high temperature as described [Bartolomé et al., 2007]. Briefly, for the amplification of the genomic HCV-RNA strand, the cDNA synthesis was carried out at 65ºC for 20 minutes with 0.5 µg of total RNA isolated from liver or PBMCs, or with 5 µL of total RNA isolated from 250 µL or 2 mL of serum, in 20 µL reaction mixture containing 0.5 µmol/L of the antisense primer (UTRLC2: 5'-CAAGCACCCTATCAGGCAGT-3'), 1 mmol/L $MnCl_2$, 200 µmol/L of each deoxynucleotide triphosphate, 1× RT buffer (Applied Biosystems, Foster City, CA, USA) and 5 units of Tth (Applied Biosystems).. For the detection of the antigenomic HCV-RNA strand in the liver biopsies of the patients, the cDNA was synthesized under the same conditions but adding the sense primer (UTRLC1: 5'-CTTCACGCAGAAAGCGTCTA-3') instead of the antisense primer.

Real-time PCR was performed with the fluorescence resonance energy transfer (FRET) probes described by Bullock et al. [2002]: sensor probe (5'-LCRed640-GTACACCGGAATTGCCAGGA-phosphate-3') and anchor probe (5'-GCCATAGTGGTCGTCTGCGGAACCGGT-fluorescein isothiocyanate-3').

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PCR reactions were run in the Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) in glass capillaries in a final volume of 20 µL. The reaction mixture consisted of 2 µL of cDNA, 0.5 µmol/L of primers UTRLC1 and UTRLC2, 0.2 µmol/L of each probe and 2 µL of 10x LightCycler FastStart DNA Master HybProbe mix (Roche Molecular Biochemicals) containing nucleotides, FastStart Taq DNA polymerase and 10 mmol/L $MgCl_2$. The final $MgCl_2$ concentration in the reaction mixture was adjusted to 2 mmol/L. Each test run included a reagent blank, in which template cDNA was replaced with PCR-grade water. The amplification protocol was as follows: initial activation of the enzyme for 10 minutes at 95ºC, followed by 60 cycles of denaturation at 95ºC for 0 seconds, annealing at 55ºC for 12 seconds and extension at 72ºC for 12 seconds with a ramping rate of 20ºC/second. The fluorescence was measured at the end of each annealing phase. After amplification, a melting curve was generated by denaturing the reactions at 95ºC for 5 seconds, cooling the samples to 40ºC for 30 seconds and then slowly heating the samples to 80ºC with a temperature transition rate of 0.1ºC/second and continuous fluorescence data acquisition. Two standard curves constructed with 10-fold dilutions of synthetic genomic and antigenomic HCV-RNA were used for the quantification of both HCV-RNA strands. The sensitivity of this strand-specific real-time PCR was of 3 genome copies per reaction and the dynamics of each assay was not affected when total RNA isolated from HepG2 cells was added to the reaction [Bartolomé et al., 2007]. All procedures were performed following the recommendations of Kwok and Higuchi [1989].

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Anti-HCV detection

Anti-HCV was checked again in all patients using a commercial assay (Innotest-HCV-AbIV; Innogenetics, Ghent, Belgium). In addition, all patients were tested for the presence of IgG antibodies directed to an immunodominant HCV core-derived epitope (anti-core HCV) by an ELISA developed recently [Quiroga et al., 2009] (Spanish patent no. P200800493 and patent application no. PCT/ES/2009000019). In brief, wells of a 96-well microtitre plate (Costar, Cambridge, MA, USA) were coated with 10 μg/mL HCVcore peptide (sequence PKPQRKTKRNTNRRP corresponding to amino acids 5-19 of the HCVcore protein; GeneScript Corporation, Scotch Plains, NJ,USA) in 0.1 mol/L sodium carbonate buffer pH 9.6 for 18 h at 4ºC. Wells were washed with PBS pH 7.4 containing 0.05% Tween-20 (Sigma Chemical Co., St.Louis, MO, USA) and non-specific sites blocked (1 h, 37ºC) with PBS containing 0.05% Tween-20 plus 10% heat-inactivated fetal bovine serum (SeraLab, West Sussex, UK). Serum samples were diluted (1:10) in blocking buffer and pre-incubated for 1 h at 37 ºC with shaking; then, duplicate samples were reacted with HCVcore-coated wells for 1h at 37ºC (100 μL/well). Wells were washed five times as above and incubated (1 h, 37 ºC) with horseradish peroxidase-conjugated rabbit polyclonal anti-human IgG (Dako Cytomation A/S, Glostrup, Denmark) diluted 1:1000 in blocking buffer. After five washings wells were reacted for 30 min at room temperature in the dark with 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt (Pierce, Rockford, IL, USA) and the absorbance value measured at 405 nm with a reference at 620 nm. A sample was considered reactive to IgG anti-core HCV if the absorbance value exceeded the mean absorbance values of 20 non-exposed, HCV-negative healthy volunteers plus five times the standard deviation.

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Statistical analysis

Statistical analysis was performed using SPSS software. Categorical variables were compared by the chi-square test or the Fisher's exact test and continuous variables were compared with the nonparametric Mann-Whitney test. Correlations were done using the Spearman's correlation coefficient. All reported *P* values are two-sided.

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RESULTS

All the 122 patients with occult HCV infection included in the study as well as the 45 patients of the control group were serum HCV-RNA negative when viral RNA was tested in 250 µL of serum. Anti-HCV was also undetectable by the commercial assay in all cases.

Regarding detection of IgG anti-core HCV, all patients without HCV-RNA in the liver (control group) were negative for this marker while 36% (44/122) of the patients with occult HCV were positive. Titration of anti-core HCV showed a median value of 1:20 and antibodies titers ranged from 1:10 to 1:160. Levels of liver enzymes, liver inflammation, liver fibrosis and genomic and antigenomic HCV-RNA loads in liver were similar between patients with and without anti-core HCV, and the antibody titers did not correlate with any parameter (data not shown).

After ultracentrifugation of serum samples, HCV-RNA was found in 70/122 (57%) patients with occult HCV with a median of 70.5 copies/mL (range: 20-192 copies/mL), while all patients of the control group were HCV-RNA negative. The antigenomic HCV-RNA strand (HCV replication) was detected more frequently in the liver of the patients with occult HCV infection who were serum HCV-RNA positive (62/70: 88%) than in those patients with occult HCV but without serum HCV-RNA (29/52: 6%; $p<0.001$). No other clinical, histological or virological differences were found between these two groups of patients and serum HCV-RNA load was not correlated with any parameter (data not shown).

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HCV-RNA was positive in the PBMCs of 74/122 (61%) patients with occult HCV but in none of the control group. The median HCV-RNA load was $2.7x10^4$ copies/µg total RNA (range: $3x10^2$–$4.1x10^5$). Replication of HCV in the liver was detected more frequently (p=0.005) in patients who were HCV-RNA positive in PBMCs (62/74: 84%) than in the patients who were negative (28/48: 60%), but no other differences were found. The quantity of HCV-RNA in PBMCs did not correlate with any parameter (data not shown).

Combining two assays, the best results were achieved with the detection of HCV-RNA in ultracentrifuged serum and in PBMCs, as occult HCV infection was identified in 104/122 (85%) of the patients (Figure 1). When combining the detection of anti-core HCV and the detection of HCV-RNA in serum (after ultracentrifugation) and in PBMCs, up to 91% of the patients (111/122) were positive for at least one of these markers and so that they could be diagnosed serologically as occult HCV infection. Of these 111 positive patients, 48 (43.2%) had only one of the markers studied (7 anti-core HCV, 17 HCV-RNA in ultracentrifuged serum and 24 viral RNA in PBMCs) and 49 (44%) patients were positive for two markers (10 had anti-core HCV and HCV-RNA in PBMCs, 13 had anti-core HCV and HCV-RNA in ultracentrifuged serum, and 26 were HCV-RNA positive in ultracentrifuged serum and in PBMCs). The other 14 patients (12.6%) were positive simultaneously for the three markers.

The amount of genomic HCV-RNA in liver was significantly higher (p=0.006) in the 14 patients who were positive simultaneously for anti-core HCV and for HCV-RNA in serum and in PBMCs than in the 11 patients who were negative by

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the three assays (Table 2). The genomic HCV-RNA load in the liver of the 14 patients was also significantly higher (p=0.039) than that of patients who were positive for one or for two HCV markers (Table 2). Regarding replication of HCV in the liver, no differences were found in the antigenomic HCV-RNA loads among the groups (Table 2). However, the number of patients with detectable antigenomic HCV-RNA strand was significantly lower in those without HCV markers (3/11: 27%) than in the group of patients who were positive for one (35/48: 73%; p=0.011), two (40/49: 82% p=0.001) or three HCV markers (13/14: 93%; p=0.002). Finally, liver inflammation and liver fibrosis were observed more frequently in the patients who were positive for the three HCV markers but without significant differences (Table 2).

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DISCUSSION

Patients with occult HCV infection have HCV-RNA in the liver but anti-HCV and serum viral RNA are undetectable by routine assays [Castillo et al., 2004; Esaki et al., 2004; Comar et al., 2006]. However, these patients may have HCV-RNA in the PBMCs [Castillo et al., 2004; Zaghloul and El-Sherbiny, 2010] and HCV-RNA can be detected in the serum of 58% of the patients after concentrating circulating viral particles by ultracentrifugation [Bartolomé et al., 2007]. The development of a new ELISA has allowed the detection of IgG anti-core HCV antibodies in 31% of the patients with occult HCV infection [Quiroga et al., 2009]. This study examined the possibility of diagnosing occult HCV infection in blood samples combining the above mentioned assays.

In this study, 122 patients with a diagnosis of occult HCV infection (with detectable HCV-RNA in the liver) and 45 control patients (who did not have HCV-RNA in the liver) were enrolled. All the control patients were negative to the HCV markers studied in the present work, supporting the specificity of the assays. HCV-RNA was detected in the PBMCs of 61% of the 122 patients with occult HCV infection and viral RNA was positive in the serum of 57% of the patients after concentrating the circulating viral particles by ultracentrifugation. Although all the patients were anti-HCV negative by a commercial ELISA, anti-core HCV antibodies were detectable with the “in house” ELISA in 36% of the patients with occult HCV. This new anti-core assay may have an increased sensitivity because of the use of a single, well-conserved immunodominant peptide epitope that may facilitate binding of minute amounts of specific antibodies present in the serum of patients with occult HCV. In contrast, licensed

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anti-HCV immunoassays make use of a mixture of several recombinant HCV-derived proteins within which such a short epitope may be hidden for encounter with very small quantities of antibodies.

The positivity rates found in this study for each HCV marker are similar to those reported in the original papers [Castillo et al., 2004; Bartolomé et al., 2007; Quiroga et al., 2009]. The present results confirm that the detection of HCV-RNA in PBMCs is the best surrogate marker for the diagnosis of occult HCV, but a proportion of patients remains undiagnosed if no other marker is tested. The combination of HCV-RNA detection in ultracentrifuged serum and in PBMCs allowed the identification of occult HCV infection in 85% of the patients. Seven patients (6%) were only positive for anti-core HCV. It could be considered that this situation merely reflects an immunological response to a past exposure to HCV rather than an ongoing occult infection and therefore, this marker could not be appropriate for the diagnosis of an active occult HCV infection. This seems unlikely because all the control patients studied (without HCV-RNA in the liver), as well as those included in the original description of the assay [Quiroga et al., 2009], were negative to anti-core HCV. When combining HCV-RNA detection in PBMCs and in ultracentrifuged serum along with detection of anti-core HCV, up to 91% of the patients with occult HCV could be properly diagnosed.

It was reported by testing for HCV-RNA in PBMCs that 45% of hemodialysis patients with persistent abnormal values of liver enzymes of unknown etiology had an occult HCV infection [Barril et al., 2008]. As a liver biopsy is not recommended routinely in hemodialysis patients, the use of the assays described

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in this work may improve the diagnosis of occult HCV infection in these patients [Thongsawat et al., 2008]. Furthermore, testing for anti-core HCV and for HCV-RNA in PBMCs and in ultracentrifuged serum may be performed in other groups at risk for occult HCV infection, such as family members of patients with occult HCV infection [Castillo et al., .2009], patients with hematological disorders, patients infected with HIV, or intravenous drug addicts, as well as in blood banks because occult HCV infection has been identified in subjects without evidence of liver disease [De Marco et al., 2009].

Interestingly, the possibility of detecting these markers seems to be related to the levels of HCV infection in the liver. Thus, patients who were positive simultaneously for anti-core HCV and for HCV-RNA in PBMCs and in ultracentrifuged serum had significantly larger amount of genomic HCV-RNA in the liver than the other patients. The percentage of patients with detectable replication of HCV in the liver (presence of antigenomic HCV-RNA strand) was significantly lower in patients without detectable HCV markers (27%) than in those patients who were positive for one (73%, p=0.011), two (82%, p=0.001) or three HCV markers (93%, p=0.002). It could be assumed that when replication of HCV is detectable in the liver of these patients there may be a greater release of viral particles into the circulation, allowing the detection of HCV-RNA in serum after ultracentrifugation. Low levels of circulating viral particles may be capable of eliciting an antibody response against the viral nucleocapsid and therefore, anti-core HCV can be detected by the “in house” ELISA.

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Regarding liver damage, liver inflammation and fibrosis were observed more frequently in patients who were positive for the three HCV markers than in the rest of the patients, although without significant differences. Future studies should be performed to assess whether in patients with occult HCV, liver damage may be related to the extent of HCV infection in liver.

In summary, testing for anti-core HCV, serum HCV-RNA after ultracentrifugation and HCV-RNA in PBMCs allows diagnosis of occult HCV infection in 91% of the patients without the need of performing a liver biopsy. The feasibility of diagnosing an occult HCV infection by combining non-invasive assays in patients with chronic liver disease of unknown etiology and in other groups at risk for this infection will permit to elucidate the clinical significance of occult HCV infection that at present remains unknown.

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Disclosure of Conflicts of Interest

Authors have nothing to disclose.

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FIGURE LEGEND

Figure 1 Group A. Detection of anti-core HCV and of HCV-RNA in serum (after ultracentrifugation): 17 patients with anti-core HCV alone, 43 with serum HCV-RNA alone and 27 patients positive simultaneously for both markers. Group B. Detection of anti-core HCV and of HCV-RNA in PBMCs: 20 patients with anti-core HCV alone, 50 patients with HCV-RNA in PBMCs alone and 24 patients with both markers. Group C: Detection of HCV-RNA in serum (after ultracentrifugation) and in PBMCs: 30 patients with serum HCV-RNA alone, 34 patients with HCV-RNA in PBMCs alone and 40 patients with both markers. Group D. Detection of anti-core HCV and of HCV-RNA in PBMCs and in serum (after ultracentrifugation): 48 patients positive for one HCV marker, 49 patients with two markers and 14 positive simultaneously for the three markers.

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Table 1 Characteristics of the patients with occult HCV infection

	Patients with occult HCV infection (n = 122)
Gender (M/F)	90/32
Age (yr.)[a]	45.0 (42.9-47.1)
Known duration of disease (mo.)[a]	69.8 (56.3-83.2)
Load of genomic HCV-RNA in liver (copies/μg total RNA)[b]	$2.4x10^5$ ($3.2x10^3$-$3.2x10^6$)
Antigenomic HCV-RNA in liver[c]	91 (75)
Load of antigenomic HCV-RNA in liver (copies/μg total RNA)[b]	$4.7x10^4$ ($4.0x10^2$-$1.0x10^6$)
AST (IU/L)[a]	38.6 (34.8-42.4)
ALT (IU/L)[a]	78.2 (66.2-90.3)
GGTP (IU/L)[a]	93.6 (76.9-110.3)
Liver inflammation[c]	53 (43)
Fibrosis[c]	36 (29)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGTP: Gamma-glutamyl transpeptidase (normal values for AST and ALT, <40 IU/L; normal values for GGTP, <45 IU/L). [a]Expressed as mean (95% confidence interval of the mean). [b]Expressed as median (range). [c]Expressed as number of cases (%)

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Table 2 Characteristics of the patients with occult HCV infection according to the status of HCV markers (anti-core HCV, HCV-RNA in PBMCs and HCV-RNA in ultracentrifuged serum)

	Negative markers (n = 11)	One positive marker (n = 48)	Two positive markers (n = 49)	Three positive markers (n = 14)
Load of genomic HCV-RNA in liver [1](copies/µg total RNA)	[(a)]$1.2x10^5$ ($2.0x10^4$-$6.4x10^5$)	[(b)]$2.5x10^5$ ($2.5x10^4$-$2.3x10^6$)	[(b)]$2.3x10^5$ ($3.2x10^3$-$3.2x10^6$)	[(c)]$4.8x10^5$ ($1.4x10^5$-$1.7x10^6$)
[2]Antigenomic HCV-RNA in liver	[(d)]3 (27)	[(e)]35 (73)	[(f)]40 (82)	[(g)]13 (93)
Load of antigenomic HCV-RNA in liver [1](copies/µg total RNA)	$3.3x10^4$ ($2.5x10^3$-$1.0x10^5$)	$2.3x10^4$ ($4.0x10^2$-$1.0x10^6$)	$3.6x10^4$ ($9.8x10^2$-$5.2x10^5$)	$7.8x10^4$ ($1.5x10^3$-$6.2x10^5$)
AST [3](IU/L)	36.5 (27.8-45.3)	40.9 (33.6-48.2)	37.6 (31.8-43.5)	35.7 (26.4-45.1)
ALT [3](IU/L)	62.4 (43.7-81.1)	88.1 (67.3-108.9)	76.8 (55.4-98.3)	61.8 (41.0-82.8)
GGTP [3](IU/L)	61.2 (31.6-90.8)	108.1 (77.2-139.0)	80.7 (74.8-106.2)	114.4 (68.7-160.1)
[2]Liver inflammation	2 (18)	21 (44)	22 (45)	8 (58)
[2]Fibrosis	1 (9)	15 (31)	15 (31)	5 (36)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGTP: Gamma-glutamyl transpeptidase (normal values for AST and ALT, <40 IU/L; normal values for GGTP, <45 IU/L). [1]Expressed as median (range); [2]Expressed as number of cases (%); [3]Expressed as mean (95% confidence interval of the mean). (a) *vs* (c) $P = 0.006$; (b) *vs* (c) $P = 0.039$; (d) *vs* (e) $P = 0.011$; (d) *vs* (f) $P = 0.001$; (d) *vs* (g) $P = 0.002$

For Peer Review