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# Apoptosis Markers Related to Pathogenesis of Pediatric Chronic Hepatitis C Virus Infection: M30 Mirrors the Severity of Steatosis

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Apoptosis involvement in liver damage related to hepatitis C virus (HCV) chronic infection has been suggested. Although liver biopsy represents the gold standard for evaluating disease severity, non-invasive tests are a growing medical need. The aim of this study was to detect apoptosis markers in liver and serum from pediatric HCV-infected patients and to assess its utility to predict liver damage progression. Twenty-three patients were included. Liver biopsies were used for histological analysis as well as for immunodetection of a viral protein (NS3) and apoptosis markers (activated caspase-3 [casp-3a], caspase-generated CK-18 fragment [M30] and TUNEL). M30 was quantified in paired serum and biopsy samples. NS3 correlated both with casp-3a ($r = 0.83$, $P < 0.0001$) and TUNEL ($r = 0.61$, $P < 0.0017$). Casp-3a and TUNEL also displayed a correlation ($r = 0.56$, $P = 0.005$). Both NS3 and casp-3a were associated with fibrosis stage ($P = 0.03$). Serum M30 [median: 122.15 UL-1 (86.68–794.58)] was higher in patients than in controls [median: 81.44 UL-1 (41.17–129.30)], ($P < 0.0001$). M30 showed a correlation with steatosis, and indeed it was linked to severe grade ($P = 0.004$). In children, HCV would be involved in liver damage through apoptosis induction. The apoptosis markers detected reflect liver injury. Serum M30 might be useful as a marker to detect the extent of liver steatosis. ***J. Med. Virol. 82:949–957, 2010.***



**KEY WORDS:** activated-caspase-3; M30; TUNEL

## INTRODUCTION

The natural history of chronic hepatitis C virus (HCV) infection acquired in childhood remains characterized poorly and the long-term outcome is still a matter of debate. Perinatal transmission and drug abuse have become the most important routes of HCV infection in children and adolescents [Jara et al., 2003].

Hepatitis related to HCV is a progressive disease that may result in chronic active hepatitis, cirrhosis, and hepatocellular carcinoma [Lauer and Walker, 2001], but liver disease seems to be milder in children than in adults, and cirrhosis has been described rarely in the former [Badizadegan et al., 1998; Murray et al., 2005]. The mechanisms leading to liver cell injury, inflammation, steatosis, and fibrosis are not fully understood.

Apoptosis plays a major role in development and tissue homeostasis in addition to pathological processes [Wyllie et al., 1980]. It has been demonstrated that hepatocyte apoptosis plays a role in liver pathogenesis of hepatitis C; as well as it may be associated with liver fibrogenesis [Rust and Gores, 2000; Bantel and Schulze-Osthoff, 2003; Fischer et al., 2007]. Most of the nuclear and cytoplasmic morphological changes of apoptotic cells are due to caspases, a group of cysteine proteases that cleave substrates after aspartate residues [Cohen, 1997]. DNA fragmentation is recognized as an apoptotic late event, whereas caspase activation measurement is a suitable marker for detecting early signs of liver apoptosis. Indeed, it has been reported that activated caspase or caspase-cleaved substrates are detected in hepatocytes without late apoptotic features [Bantel et al., 2001].

Proteins of the cytokeratin type I family are caspase substrates whose cleavages contribute to cellular collapse during apoptosis. Cytokeratin-18 (CK18) is the major intermediate filament in liver cells. It is cleaved by caspases at two conserved residues during apoptosis,

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one of them (Asp 396) represents a neoepitope (M30), which is not detectable in vital or necrotic cells but is enhanced in various liver diseases [Leers et al., 1999]. Although liver biopsy represents the gold standard for evaluating disease severity and fibrosis stages, it is an invasive technique with inherent risks that can be repeated only at infrequent intervals. Therefore, the development of non-invasive tests that can accurately predict initial stage of disease and fibrosis progression represents a high priority and a growing medical need [Afdhal and Nunes, 2004]. In recent years, there has been increasing interest in non-invasive assessment of liver fibrosis by use of surrogate indirect serum markers such as aminotransferases, aspartate aminotransferase (AST)-to-platelet ratio (APRI) and AST-to-alanine aminotransferase (ALT) ratio (AAR), but they reflect alterations in hepatic function rather than extracellular matrix metabolism. Since several reports described normal aminotransferase levels in about 25–30% of chronically HCV-infected pediatric patients [Kage et al., 1997; Jara et al., 2003; Gismondi et al., 2004; Sanai et al., 2008; Sebastiani et al., 2008] there may be a potential advantage in the detection of serum direct markers that do not involve transaminases. Bantel et al. [2004b] have identified M30 as a sensitive biomarker based on a rationale understanding of cell biology for detecting early apoptotic liver injury associated to HCV in adults.

The aim of the present study was to investigate the role of apoptosis in pediatric chronic HCV infection by means of the detection of apoptosis markers both in liver biopsy and serum.

## PATIENTS AND METHODS

### Patients

Twenty-three pediatric patients with chronic HCV infection [median age: 6 years (1–17 years); 65.2% female] who attended the Liver Unit at the Ricardo Gutierrez Children's Hospital, University of Buenos Aires were included in this retrospective study. Diagnosis was based on the presence of anti-HCV antibodies in serum at or after 18 months of age and HCV RNA in plasma at one or more separate occasions. HCV genotype was assessed according to Simmonds et al. [1993]. Serum AST and ALT levels, and body mass index (BMI) were obtained from clinical records.

Patients had no other causes of liver disease; cases with autoimmune or metabolic disorders and coinfection with hepatitis B virus and/or human immunodeficiency virus were excluded. Informed consent was obtained from parents of all the included patients and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's ethics and research committees.

Clinical, virological, and histological features of patients are described in Table I.

### Samples

Formalin-fixed and paraffin-embedded liver samples were used for histological analysis as well as for immunodetection of a viral protein and different apoptosis markers. Serum samples at time of biopsy for M30 assessment were selected from the HCV positive serum archives of the Laboratory of Molecular Biology, Pathology Division, Ricardo Gutiérrez Children's Hospital. Serum samples were stored frozen at −80°C.

### Histological Analysis

Review of biopsies was performed by two pathologists. Grading of necroinflammatory activity and staging of fibrosis were semiquantitatively assessed using the modified Knodell scoring system (HAI) and METAVIR [Theise et al., 2007]. Each biopsy specimen was categorized with a diagnosis of minimal (≤3), mild (4–6), moderate (7–12), or severe (>12) HAI. Presence of lymphoid follicles as well as of bile duct lesion and grade of steatosis were also evaluated. Steatosis was graded as follows: minimal (1–33% of hepatocytes affected), moderate (>33–66%), or severe (>66%).

### Immunohistochemical Analysis (IHC).

Tissue sections were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol. For optimal epitope retrieval, sections were treated with sodium citrate buffer (0.01 M, pH 6). Endogenous biotin was blocked with Biotin Blocking System (DakoCytomation, Carpinteria, CA). Sections were then incubated with primary antibodies to: HCV NS3 protein (NS3) (Biogenex, San Ramon, CA), caspase-3-mediated cleavage generated neoepitope of CK18 (M30) (Roche, Mannheim, Germany) and activated caspase-3 (R&D Systems, Minneapolis, MN). Immunohistochemical staining was obtained by applying the streptavidin–biotin complex—peroxidase system and substrate-chromogen reagent (LSAB+System-HRP and DAB; DakoCytomation). IHC was selective for each marker, in that reactive products were not observed in the absence of the respective primary antibody. As positive controls, previously studied pediatric non-alcoholic steatohepatitis and chronic hepatitis B virus infection liver biopsy samples positive for both activated caspase-3 and M30 were used. As negative control, liver biopsy slides from 11 pediatric uninfected patients with congenital liver fibrosis, a disease without steatosis or inflammation, were included. To evaluate the specificity of anti-NS3 antibody, liver samples from pediatric patients with chronic hepatitis B virus infection were analyzed.

### TUNEL Staining

DNA fragmentation was visualized by an enzymatic reaction using ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON International, Inc., Temecula, CA) according to the manufacturer's instructions. As positive control, a female rodent mammary gland slide provided by the supplier was used, whereas the same slide without applying the TdT was used as negative control.

TABLE I. Clinical, Virological, and Histological Features of HCV Patient

| Patients | | Clinical and serological characteristics | | | | | | | | Histological characteristics* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Transaminases | | | | | | |
| | | Sex | Ages (years) | Risk factor for HCV infection | Genotype | BMI | AST, UL-1 | ALT, UL-1 | AST/ALT, ratio | Knodell | Lymphoid follicle | Bile duct damage | Steatosis (%) |
| 1 | BxI | M | 3 | T | 1a | 15.41 | 69 | 57 | 1.21 | 6 (5+1) | No | Yes | 15 |
| | BxII | | 5 | | | 15.54 | 34 | 40 | 0.85 | 10 (8+2) | No | Yes | 20 |
| | BxIII | | 13 | | | 19.27 | 47 | 43 | 1.09 | 8 (5+3) | No | No | 0 |
| 2 | | F | 14 | T | 1b | 20.23 | 55 | 86 | 0.64 | 11 (9+2) | No | No | <10 |
| 3 | | F | 4 | V | 1a/c | 14.99 | 46 | 34 | 1.35 | 10 (7+3) | Yes | Yes | 0 |
| 4 | | F | 17 | T | 1a/c | 17.08 | 39 | 43 | 0.91 | 8 (4+4) | No | Yes | 50 |
| 5 | | M | 4 | V | 1a/c | 17.09 | 84 | 97 | 0.86 | 10 (9+1) | Yes | Yes | 80 |
| 6 | BxI | F | 3 | V | 1a/c | 18.38 | 26 | 19 | 1.37 | 10 (7+3) | Yes | No | 0 |
| | BxII | | 6 | | | 21.42 | 13 | 11 | 1.18 | 7 (3+4) | No | No | 0 |
| | BxIII | | 13 | | | 27.95 | 23 | 21 | 1.09 | 8 (5+3) | No | Yes | <10 |
| 7 | | F | 16 | Unknown | 1a/c | 22.42 | 30 | 41 | 0.73 | 12 (10+2) | Yes | Yes | 15 |
| 8 | BxI | M | 3 | V | 1a/c | 17.34 | 71 | 91 | 0.78 | 6 (5+1) | Yes | Yes | 15 |
| | BxII | | 6 | | | 24.17 | 314 | 364 | 0.86 | 11 (8+3) | No | Yes | 85 |
| 9 | | F | 6 | V | 1a/c | 14.44 | 35 | 50 | 0.70 | 8 (7+1) | Yes | Yes | 20 |
| 10 | | F | 8 | Unknown | 1a | 16.96 | 41 | 38 | 1.08 | 9 (7+2) | Yes | Yes | 0 |
| 11 | BxI | M | 6 | Unknown | ND | 14.93 | 14 | 17 | 0.82 | 11 (8+3) | No | Yes | 0 |
| | BxII | | 13 | | | 19.76 | 56 | 71 | 0.79 | 10 (7+3) | No | Yes | 0 |
| 12 | | F | 17 | V | 1a/c | 21.84 | 21 | 11 | 1.91 | 6 (3+3) | No | Yes | <10 |
| 13 | | F | 3 | V | 1a/c | 18.28 | 84 | 137 | 0.61 | 6 (5+1) | No | Yes | 60 |
| 14 | | F | 3 | V | 1b | 16.41 | 65 | 75 | 0.87 | 9 (6+3) | Yes | Yes | 40 |
| 15 | | F | 1 | V | 4 | 17.74 | 57 | 33 | 1.73 | 11 (7+4) | Yes | Yes | 0 |
| 16 | | F | 17 | T | 1a/c | ND | 22 | 16 | 1.37 | 10 (7+3) | No | Yes | 10 |
| 17 | | M | 1 | T | 1b | 14.49 | 159 | 213 | 0.75 | 14 (11+3) | Yes | Yes | 10 |
| 18 | | F | 8 | T | 1b | 14.36 | 10 | 12 | 0.83 | 6 (5+1) | No | No | 0 |
| 19 | | M | 15 | T | ND | 19.58 | 58 | 76 | 0.76 | 16 (10+6) | No | Yes | 0 |
| 20 | | F | 6 | V | 1a/c | 19.97 | 56 | 55 | 1.02 | 6 (5+1) | No | Yes | 70 |
| 21 | | M | 15 | T | 1b | 18.36 | 20 | 24 | 0.83 | 13 (10+3) | Yes | Yes | 0 |
| 22 | | M | 12 | Unknown | 1a/c | 29.58 | 83 | 113 | 0.73 | 11 (8+3) | No | Yes | 30 |
| 23 | BxI | F | 13 | T | 1a/c | 19.68 | 62 | 65 | 0.95 | 14 (11+3) | Yes | Yes | 10 |
| | BxII | | 15 | | | 20.28 | 113 | 75 | 1.51 | 12 (9+3) | No | No | 40 |
| | BxIII | | 16 | | | 20.31 | 22 | 18 | 1.22 | 11 (8+3) | No | Yes | 60 |

*Knodell scoring system: Grading of necroinflammatory activity and staging of fibrosis.
F, female; M, male; BMI, body mass index; ND, not determined; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Risk factor for HCV infection: T, transfusion; V, maternal HCV infection.
Normal ALT and AST levels were $\leq$32 and $\leq$48 IUL-1, respectively when testing was done at 37°C.
BxI, BxII, BxIII denote: multiple liver biopsies.

### Quantification

IHC and TUNEL-stained sections were examined by light microscopy and positive cells were counted. Twenty high-power fields were selected on the basis of the best-preserved tissue areas. The immunoreactivity was expressed as the number of positive hepatocytes/number of total hepatocytes in 20 high-power fields (1,000×).

### ELISA

Serological caspase activity was quantify with a novel M30-Apoptosense ELISA kit (PEVIVA AB, Bromma, Sweden), using the same antibody applied for immunohistochemistry, to detect the caspase-generated neoepitope of the CK-18 proteolytic fragment (M30). Assays were performed in duplicate according to the manufacturer's instructions. Pre-surgical sera from 16 pediatric otherwise healthy patients who underwent bone surgery were used as controls.

### Statistical Analysis

Statistical analysis was performed using GraphPad InStat software, version 3.05 (Graphpad, San Diego, CA). Mann–Whitney *U*-test and unpaired *t*-test were used to compare sets of data. Pearson's and Spearman's correlations were applied when appropriate. $P < 0.05$ values were considered significant. Receiver-operating characteristic (ROC) plot analysis was used to estimate cut-off values of serum M30 with optimal sensitivity and specificity. Cut-off values were determined using the MedCalc demo statistical software (MedCalc Software, Mariakerke, Belgium).

## RESULTS

### Clinical and Liver Biopsy Findings

HCV genotype 1 was present in 87% of cases and only one patient displayed genotype 4. The risk factors for HCV infection were maternal HCV infection in 43.5% of patients (10/23), transfusion in 39.1% (9/23), and unknown in 17.4% (4/23). According to age- and sex-specific NCHS/CDC2000 BMI values (http://www.cdc.gov/growthcharts, 2000), seven patients (31.8%) showed elevated BMI (four [18.2%] overweighed and three [13.6%] obese). The AST and ALT levels at time of biopsy (considering multiple biopsies from the same patient) were elevated in 48.4% (15/31) and 71% (22/31) serum samples, respectively. Eighty percent of biopsies showed moderate or severe HAI. Concerning fibrosis, bridging fibrosis (stage 3) was predominant among studied biopsies (51.6%). The only one case which displayed cirrhosis was a thalassemia patient. Lymphoid follicles, characteristic of chronic HCV infection in adults, were present in 38.7% specimens, whereas bile duct lesions were observed in 80.6%. Hepatocellular fat accumulation, typically a mixture of small and large droplet fat, was present in 64.5% of biopsy specimens; however, only 9.7% showed severe steatosis.

Aminotransferase values showed no statistically significant differences either among HAI groups (AST $P = 0.22$ and ALT $P = 0.22$) as well as among fibrosis stages (AST $P = 0.60$ and ALT $P = 0.25$).

### HCV NS3 Detection in Liver Biopsies

In order to determine the percentage of infected hepatocytes, HCV NS3 protein was detected by IHC in liver sections. The majority of cases (88%) displayed less than 60% of positive hepatocytes, which were not uniformly distributed (Fig. 1A). NS3 immunostaining quantification ranged from 0.004 to 0.88 (median: 0.21). No significant correlation with serum aminotransferases (AST $P = 0.93$, ALT $P = 0.55$), steatosis ($P = 0.49$) or inflammatory grade ($P = 0.63$) was found; however a weak correlation with AST/ALT ratio ($r = 0.41, P = 0.04$) and an association with fibrosis stage ($P = 0.03$) were observed (Fig. 2A).

### Detection of Early Apoptosis in Liver Tissue Sections

On H&E-stained sections, non-apoptotic cells were identified by their characteristic features of nuclear and cytoplasmic condensation. Activated caspase-3 quantification ranged from 0 to 0.133 (median: 0.056); positive biopsies (96%) displayed hepatocyte nuclear labeling (Fig. 1B). Apoptotic cells stained with anti-M30 showed variable granular cytoplasmic staining intensities in 65% of biopsy specimens. M30 quantification ranged from 0 to 0.12 (median: 0.007) (Fig. 1C).

Both early apoptotic markers did not correlate with biochemical parameters (AST, ALT, and AST/ALT ratio). Activated caspase-3-labeled hepatocytes displayed a statistically significant correlation with HCV positive cells ($r = 0.83$, $P < 0.0001$) (Fig. 2B). Higher numbers of activated caspase-3-labeled cells were associated with worse fibrosis stages ($P = 0.03$) (Fig. 2A), but no association was observed with steatosis ($P = 0.30$), bile duct lesion ($P = 0.20$) and lymphoid follicles ($P = 0.98$). An increased number of M30 labeled hepatocytes was detected in those biopsies without lymphoid follicles ($P = 0.05$) as well as without bile duct lesion ($P = 0.05$), but it did not show association either with steatosis ($P = 0.63$) or with fibrosis ($P = 0.44$).

Activated caspase-3 and M30 correlation was not statistically significant ($P = 0.68$), albeit an excess of activated caspase-3 positive cells respect to M30 positive ones was observed in 72% of HCV-infected biopsies.

Liver control samples exhibited minimal M30 or activated caspase-3 positive cells.

### Detection of Late Apoptosis in Liver Tissue Sections

TUNEL-stained hepatocytes ranging from 0 to 0.31 (median: 0.057) were wide spread scattered without a specific location pattern (Fig. 1D). TUNEL showed significant correlation with number of infected hepatocytes ($r = 0.61$, $P = 0.0017$). Early (activated caspase-3)

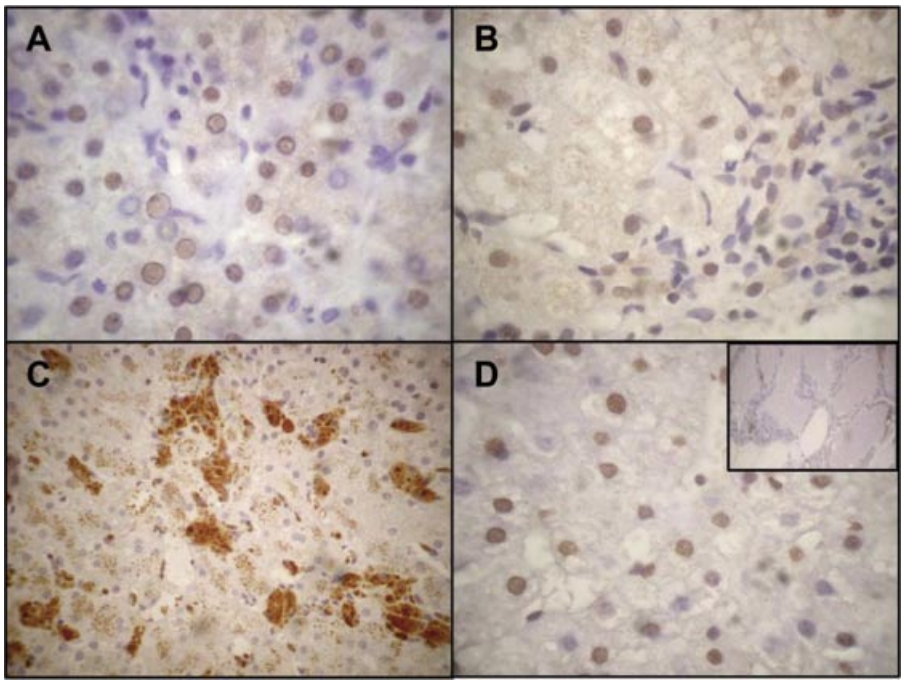


Fig. 1. Immunostaining of liver biopsies of chronically HCV-infected pediatric patients. **A**: NS3 nuclear staining in infected hepatocytes (1,000×). **B**: Activated caspase-3 positive cells display nuclear staining in periportal hepatocytes (1,000×). **C**: M30 granular cytoplasmic staining with variable intensities (400×). Note that activated caspase-3 and M30 positive cells were not overtly morphologically apoptotic. **D**: DNA fragmentation assessed by TUNEL. The nucleus of TUNEL positive cells is in dark tone (1,000×). Inset shows TUNEL positive control (400×).

and late apoptosis markers displayed statistical correlation ($r = 0.56$, $P = 0.005$). In 56% of biopsies, activated caspase-3 was higher than TUNEL quantification, which further supports the idea that immunohistochemical detection of caspase activation marks early events in the apoptosis process.

### Detection of M30 in Serum Samples

HCV patients with different grades of disease activity revealed significantly elevated serum M30 levels [median: 122.15 UL-1 (86.68–794.58)]. In contrast, healthy controls disclosed low M30 levels in serum [median: 81.44 UL-1 (41.17–129.30)], ($P < 0.0001$) (Fig. 3A). Cut-off value corresponding to the highest accuracy value (minimal false negative and false positive results) was 94.23 UL-1 (95% sensitivity, 93% specificity, positive predictive value: 95, negative predictive value: 93) (Fig. 3B). M30 serum levels were elevated in patients with severe steatosis [median: 162.35 UL-1 (120.65–794.58)] compared with patient with moderate steatosis [median: 123.65 UL-1 (98.96–243.31)], minimal or no steatosis [median: 113.70 UL-1 (86.68–279.47)] ($P = 0.004$) (Fig. 4A). Neither serum M30 nor aminotransferase levels showed statistically significant correlation with the extent of liver fibrosis (serum M30 $P = 0.63$, AST $P = 0.84$, and ALT $P = 0.48$), in contrast to intrahepatic caspase-3 activation results.

It is well known that approximately 25–30% of HCV patients have normal serum aminotransferase levels, even though most of these patients show histological evidence of chronic liver damage [Sanai et al., 2008]. Consistent with literature, 30% of the studied patients had normal aminotransferase levels at time of biopsy. Patients with elevated aminotransferase levels displayed higher serum M30 values [median: 162.35 UL-1 (98.96–794.58)] than patients with normal levels [median: 105.64 UL-1 (86.68–127.20)] (Fig. 4B).

## DISCUSSION

Apoptosis has been implicated in the pathogenesis of a number of hepatic disorders, including viral hepatitis, autoimmune diseases, non-alcoholic steatohepatitis, alcohol-induced injury, cholestasis, and hepatocellular cancer [Patel and Gores, 1995; Thompson, 1995; Que and Gores, 1996; Ghavami et al., 2005]. There is increasing evidence suggesting that liver cell damage in chronic HCV infection is mediated by apoptosis induction, which has been proposed in view of pathomorphologic features of individual infected hepatocytes [Kerr et al., 1979; Que and Gores, 1996; Bantel and Schulze-Osthoff, 2003; Bantel et al., 2004a]. However, the relative contribution of apoptosis or necrosis as well as the functional role of caspase in liver damage is largely unknown. Apoptosis of infected cells may be viewed as a cellular defense mechanism to prevent viral propagation; however, to circumvent this defense and diminish the apoptotic response, certain viruses have developed mechanisms to disrupt the normal regulation

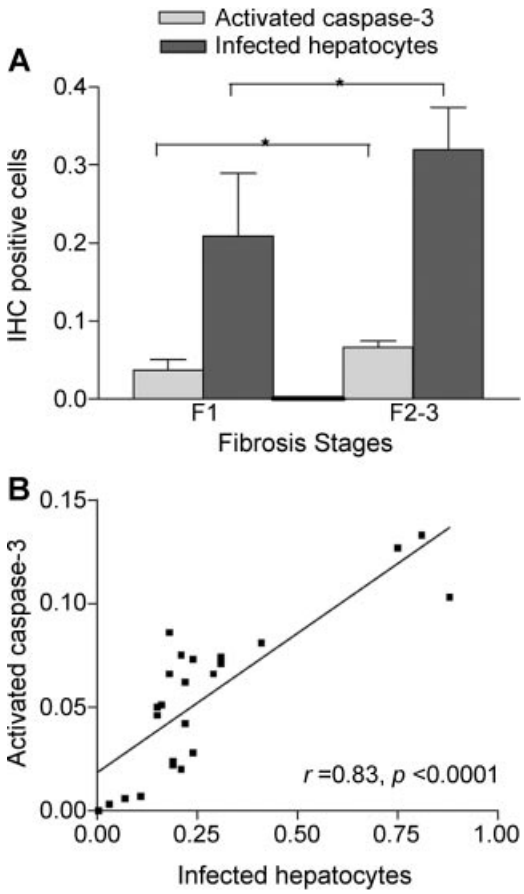


Fig. 2. Infected hepatocytes related to apoptosis markers as well as fibrosis stages. **A**: Infected hepatocytes (NS3 positive hepatocytes) and early apoptosis marker (activated caspase-3 positive cells) versus fibrosis stages. Results are expressed as mean ± SEM. Fibrosis stages according to METAVIR; $^{*}P = 0.03$. **B**: Regression analysis between infected hepatocytes and early apoptosis marker.

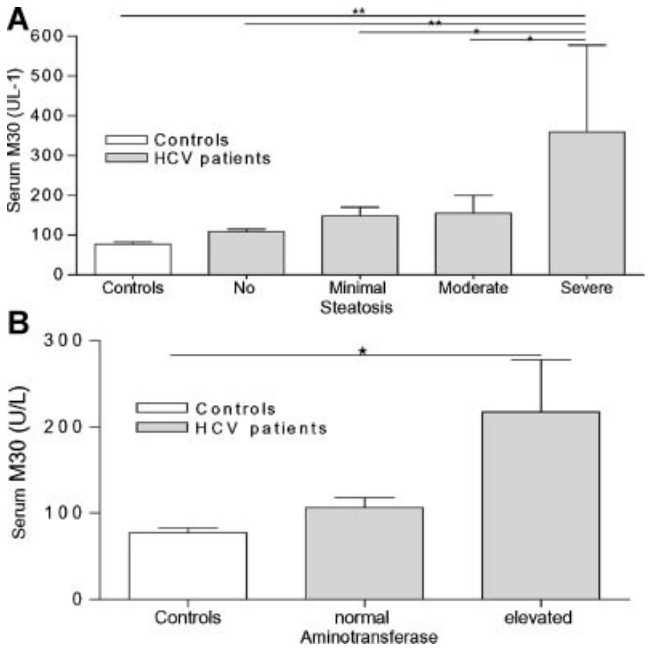


Fig. 4. Serum M30 related to histological and biochemical parameters. **A**: Serum M30 in healthy controls and patients with different grades of liver steatosis. $^{*}P < 0.05$, $^{**}P < 0.001$. **B**: Serum M30 in controls and patients with normal and elevated serum aminotransferase levels; $^{*}P < 0.001$. Results are expressed as mean ± SEM.

of death within the infected cell. Several viral proteins display either apoptotic or antiapoptotic features according to the HCV model under study [Fischer et al., 2007; Mengshol et al., 2007]. The present study evaluates apoptosis contribution to the pathogenesis of HCV infection, and correlates apoptosis with clinical, biochemical, and histological data.

Apoptosis may be assessed by different approaches. The conventional morphologic method (H&E staining), which allows the identification of the terminal stages of apoptosis (Councilman Bodies), is used rarely for quantification purposes due to low number of appreciable events; however, to overcome this problem TUNEL method was developed. In the studied series an unexpectedly high number, together with a wide distribution of late apoptotic cells were observed by TUNEL. Perhaps, this observation is due to restrictions

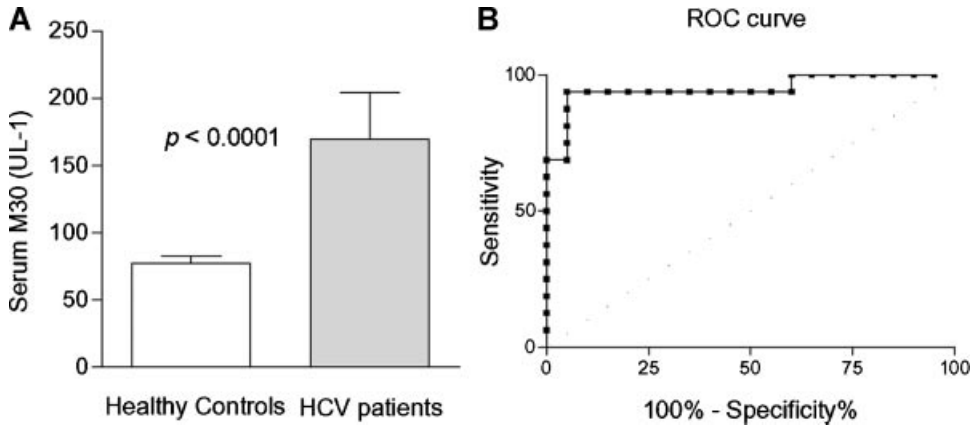


Fig. 3. M30 was increased in serum samples from HCV patients compared with healthy controls. **A**: Serum M30 (UL-1) in HCV-infected patients and healthy controls. Results are expressed as mean ± SEM. **B**: The receiver-operating characteristics (ROC) curve for M30.

of this method which cannot differentiate between apoptosis and necrosis as well as to its particular sensitivity to fixation conditions and conservation. Other biopsy markers like caspase enzymes and cytoskeleton neoepitopes seem particularly important for early apoptosis detection. When comparing TUNEL with activated caspase-3 labeling, 56% of cases in the studied series showed an excess of activated caspase-3-labeled cells, in concordance with Bantel et al. [2001] findings using double staining. The latter may be explained by the different time course of biochemical events in apoptosis: DNA fragmentation is a late event, whereas caspase activation is a reversible earlier outcome. Moreover, caspase activation may not necessarily lead to cell death; the hepatocytes may either undergo apoptosis or survive [Nhan et al., 2006].

On the other hand, it has been published that in liver biopsies of viral hepatitis and steatohepatitis there were more activated caspase-3-labeled cells than M30 ones [McPartland et al., 2005; Valva et al., 2008], as it was observed in 72% of this HCV pediatric series. Actually, it is well known that activated caspase-3 participates in other diverse biological processes, such as regulating immune response [Schwerk and Schulze-Osthoff, 2003] and in addition it has other substrates besides CK18 [Fischer et al., 2003].

It has been reported that liver injury in adult chronic HCV infection is not related to either the number of infected hepatocytes or the serum HCV viral load [Rodríguez-Iñigo et al., 1999]. Bantel et al. [2001, 2004a] have described a lack of correlation between caspase-3 activation and HCV viral load, and have postulated that caspase-3 activation is associated to liver damage. When studying these pediatric biopsies, a relation between infected hepatocytes and apoptosis markers as well as fibrosis stages was found. According to the present results HCV would not have an effect per se on histological variables; instead it would be involved in liver damage through apoptosis induction, which in turn would contribute to fibrosis development.

Liver biopsy has been accepted as the "gold standard" to assess the presence, type and stage of fibrosis and to characterize necroinflammation. However, despite its widespread use, increasing interest in hepatology converged to develop and validate non-invasive biomarkers for liver damage in hepatic diseases since it is further to be ideal [Bantel et al., 2004a; Wieckowska et al., 2006]. Recently, caspase-generated CK-18 fragment (M30) was tested in liver and in plasma from patients with liver diseases such as HCV or non-alcoholic fatty liver disease [Bantel et al., 2004a; Seidel et al., 2005; Volkmann et al., 2006; Wieckowska et al., 2006; Yilmaz et al., 2007; Diab et al., 2008]. These studies showed that M30 could be used potentially as a non-invasive diagnostic tool to predict the severity of liver diseases. Lack of similar data in HCV-infected pediatric patients prompt us to quantify serum M30. As published in adult series [Bantel et al., 2004a], the present study demonstrates that M30 was markedly increased in serum samples of HCV-infected pediatric patients and confirms its low presence under normal conditions.

It has been reported that steatosis is more frequent in HCV-infected patients than in general population meaning that HCV directly causes steatosis [Mengshol et al., 2007]. Consistent with that, 64.5% of the studied biopsies displayed steatosis. Lipid accumulation might also trigger the generation of lipid peroxidation products (pro-apoptotic), which lead to mitochondrial dysfunction, caspase activation, and cell death. In this regard serum M30, a serological marker of caspase activation, showed correlation with the grade of steatosis in the biopsy with a particular high relationship in patients with severe steatosis. The last reinforces a previous report which states that caspase activation is a prominent feature of HCV infection which is associated with steatosis [Seidel et al., 2005].

Although a remarkable activation of caspase both in biopsy and serum was observed, interesting differences between the two sample types were found: serum M30 did not mirror early apoptotic markers in liver biopsies. A plausible explanation could be that apoptotic cells are eliminated rapidly and therefore could avoid detection in liver biopsy [Bantel et al., 2004a]. Furthermore, although the exact mechanism leading to the secretion of M30 into the blood has yet to be determined, it is well known that M30 is released in serum as a consequence of necrosis secondary to apoptosis [MacFarlane et al., 2000].

It is well known from numerous studies that no quantitative correlation can be assessed between histological disease severity and biochemical parameters. Furthermore, most chronically HCV-infected patients with persistently normal ALT levels show histological evidence of chronic liver damage [Kronenberger et al., 2000]. In the studied group, serum aminotransferases did not correlate with histological liver injury whereas serum M30 seemed to be a predictor for steatosis. As it has been reported for adult patients with normal aminotransferases [Bantel et al., 2004a], in this pediatric series a link between elevated serum M30 and the most severe fibrosis stages was found, which should be confirmed in a larger pediatric cohort. These findings point out the usefulness of serum M30 as a marker of liver damage in pediatric patients.

Eventually, controversial results have been published about the effect of steatosis on virological response. However, it is well accepted that steatosis impacts on fibrosis progression, so steatosis should not be considered a benign feature, but rather a silent killer [El-Zayadi, 2008]. Although the mechanisms by which steatosis may lead to worsening fibrosis are not understood, the factors implicated include oxidative stress, insulin resistance, and increased apoptosis leading to activation of hepatic stellates cells. Evidence in favor of the latter mechanism comes from the observation that caspase activity correlates with steatosis activity and is increased in HCV infection [Walsh et al., 2004; Murray et al., 2005; Negro, 2006; Zeuzem et al., 2006; Cross et al., 2009].

Even though the relative small patient number, the novel aspect of this report is the analysis of a pediatric cohort, because data on children with chronic HCV infection are very limited. Furthermore, since HCV infection blood screening implementation, the pediatric case number has decreased. This report represents the first analysis of apoptotic markers both in biopsy and serum performed on pediatric chronic HCV patients. It would be useful to study larger pediatric cohorts, perhaps in a multicenter study, to validate the findings of this report and, in consequence, to consider caspase inhibitors as potential therapeutic strategies to prevent liver damage progression. On the other hand, serum quantitation of M30 would be useful to establish cut-off values for healthy and HCV-infected groups that would allow to follow-up the histological course of liver pathology by means of a less invasive tool than the current "gold standard" biopsy.

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