Distinctive intrahepatic characteristics of paediatric and adult pathogenesis of chronic hepatitis C infection

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Abstract

Mechanisms leading to liver damage in chronic hepatitis C (CHC) are being discussed, but both the immune system and the virus are involved. The aim of this study was to evaluate intrahepatic viral infection, apoptosis and portal and periportal/interface infiltrate in paediatric and adult patients to elucidate the pathogenesis of chronic hepatitis C. HCV-infected, activated caspase-3^+ and $TUNEL^+$ hepatocytes, as well as total, $CD4^+$, $CD8^+$, $Foxp3^+$ and $CD20^+$ lymphocytes infiltrating portal and periportal/interface tracts were evaluated in 27 paediatric and 32 adult liver samples by immunohistochemistry or immunofluorescence. The number of infected hepatocytes was higher in paediatric than in adult samples (p 0.0078). In children, they correlated with apoptotic hepatocytes (activated caspase-3^+ $r = 0.74$, $p < 0.0001$; $TUNEL^+$ $r = 0.606$, p 0.0017). Also, infected ($p = 0.026$) and apoptotic hepatocytes ($p = 0.03$) were associated with the severity of fibrosis. In adults, activated caspase-3^+ cell count was increased in severe hepatitis ($p = 0.009$). Total, $CD4^+$, $CD8^+$ and $Foxp3^+$ lymphocyte count was higher in adult samples ($p < 0.05$). Paediatric $CD8^+$ cells correlated with infected ($r = 0.495$, p 0.04) and $TUNEL^+$ hepatocytes ($r = 0.474$, $p = 0.047$), while adult ones correlated with activated caspase-3^+ hepatocytes ($r = 0.387$, p 0.04). In adults, $CD8^+$ was associated with hepatitis severity ($p < 0.0001$) and correlated with inflammatory activity ($CD8^+$ $r = 0.639$, p 0.0003). HCV, apoptosis and immune response proved to be involved in CHC pathogenesis of both paediatric and adult patients. However, liver injury in paediatric CHC would be largely associated with a viral cytopathic effect mediated by apoptosis, while in adults it would be mainly associated with an exacerbated immune response.

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Introduction

Hepatitis related to hepatitis C virus (HCV) is a progressive disease that may result in chronic active hepatitis, cirrhosis and hepatocellular carcinoma. It is estimated that about 160 million individuals (i.e. 2.35% of the world's population) are chronically infected with HCV [1,2]. The virus represents a global health problem because there is no available vaccine and, although there are recently approved direct-acting antiviral agents (DAA) available as well as more drugs in the pipeline, response to the current standards of care therapy (pegylated interferon–α and ribavirin) is limited. Given these facts, liver failure arising as a consequence of HCV infection is one of the most common reasons for liver transplants [1]. Liver disease seems to be milder in children than in adults; however, the natural history of HCV infection acquired in infancy and childhood remains poorly characterized and the long-term outcome of the disease is still a matter of debate [3].

The mechanisms leading to liver injury are under constant revision, but the fact that both immune system-mediated

reactions and viral cytopathic effects are involved in pathogenesis is widely accepted [4].

Based on the morphological characteristics of liver biopsies, it is currently accepted that hepatocyte damage is a result, at least in part, of apoptosis induction. Both *in vitro* studies and *in vivo* models have demonstrated the ability of HCV to induce apoptosis [4–7]. Furthermore, some studies including adult patients indicated that hepatocyte apoptosis plays a significant role in the pathogenesis of HCV infection, and is clinically recognized as liver inflammation and fibrosis [6, 8–12].

On the other hand, it is considered that the lack of an effective immune response during acute HCV infection is the main cause of chronic hepatitis development [13]. In chronic hepatitis C (CHC), the $CD4^+$ and $CD8^+$T cell response appears to be unsuccessful in peripheral blood, but contributes to the pathogenesis in the liver [14–18]. Normally, $CD8^+$ T cells are essential for infection control, as they migrate to infected tissues and mediate viral clearance. However, HCV-specific $CD8^+$ T cells in CHC patients possess a lesser capacity to proliferate and produce less IFN-γ in response to viral antigens [19]. Although the reasons for the global failure of a protective immune response against HCV is not fully understood, several plausible mechanisms have been proposed: (i) HCV escapes mutations, (ii) primary T cell failure or T cell exhaustion, (iii) impaired antigen presentation, (iv) suppression by HCV proteins, (v) impaired T cell maturation, (vi) suppression by regulatory T (Treg) cells and (vii) a tolerogenic environment in the liver [13].

Recently, Treg cells have become very important due to their contribution to the pathogenesis of various diseases, including hepatic diseases [20]. Accumulating evidence indicates that patients with chronic viral hepatitis display an increased number of Treg cells in peripheral blood or liver; however, the increment of Treg cells in CHC and their role in liver damage development is still a matter of debate [21–26].

Finally, in order to fully understand the pathogenesis of HCV further investigations are needed. The study of the pathogenesis of HCV in patient's samples instead of animal models, even taking into account interpatient variability, is crucial to explore different aspects of infection in its actual context. Moreover, because most studies are focused on adult cohorts and little is known about pathogenesis in children, the analysis of paediatric cases further enriches this study. In addition, it is known that the HCV immune response is compartmentalized (i.e. frequency and function of various cell types at the intrahepatic level differ from those at the periphery) [27]. As most studies are focused on peripheral blood immune cell composition, the analysis of the inflammatory infiltrate on fixed liver biopsies that identifies each population *in situ* provides a more comprehensive picture of their relationship with injury. Therefore, our aim was to evaluate intrahepatic infection, apoptosis and portal and periportal/interface (P-P/I) infiltrate in liver samples from paediatric and adult patients to further explore and compare CHC pathogenesis.

Materials and Methods

Patients and samples

Seventy-nine formalin-fixed and paraffin-embedded (FFPE) liver samples were included in the study. Namely, 27 paediatric and 32 adult CHC biopsy samples as well as 20 (10 of each age group) liver samples from healthy donor organ reduction for liver transplantation were collected from the archives of the Pathology Division at the Hospital de Niños Ricardo Gutiérrez and Hospital Italiano de Buenos Aires, respectively (Table 1).

Diagnosis was based on the presence of anti-HCV antibodies in serum samples at or after 18 months of age and HCV RNA in plasma on at least two separate occasions. Patients had no other causes of liver disease, autoimmune or metabolic disorders, hepatocellular carcinoma or co-infection with HBV and/or HIV. Patients were also treatment naïve. Cases of adults who consumed alcohol (men >80 g/day; women >60 g/day) were excluded.

Informed consent was obtained from each adult patient and from parents of paediatric patients. The study protocol conforms to the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments, as reflected in *a priori* approval by the Ethics in Research Committees of both institutions.

Tables S1 and S2 show in detail clinical, virological and histological features of each HCV paediatric and adult patient, respectively.

Histological analysis

Histological sections were blindly evaluated by two independent pathologists. Inflammatory activity and fibrosis were assessed using the modified Knodell scoring system (Histological Activity Index, HAI) and METAVIR [28]. According to HAI, each biopsy specimen was categorized as minimal (≤3), mild (4–6), moderate (7–12) or severe hepatitis (>12) (Table 1).

As METAVIR is a specially designed system for the analysis of HCV biopsies that it is more accurate for determining fibrosis stage and modified Knodell classified inflammatory activity and hepatitis and also provides a deeper characterization of damage localization and severity, we selected each score to quantify the corresponding parameter. Furthermore, in routine clinical practice both indexes are considered in this way, which allowed us to clearly compare our results with those of other authors and to minimize errors.

TABLE 1. Clinical, virological and histological patient features

	Paediatric patients	Adult patients
Age (years)		
median (range)	8 (3–14)	51 (38–74)
Male	10 (37.04%)	21 (65.26%)
Duration of infection (years)		
Median (range)	4 (1–14)	20 (10–25) [a, b]
Risk factor for HCV infection		
(*n*) Vertical	44.44% (12)	–
Drug abuse	–	21.88% (7)
Occupational	–	3.12% (1)
Transfusion	40.74% (11)	12.50% (4)
Unknown	14.81% (4)	62.50% (20)
Genotype (%, *n*)	1 (88.9%, 24)	1 (78.12%, 25)
	4 (3.69%, 1)	2 (12.50%, 4)
	ND (7.41%, 2)	3 (9.38%, 3)
Viral load (IU/ml)		
Median (range)	594 286 (1590–807 739)	560 609 ($1410–1.05 \times 10^7$)
ALT (IU/l)		
Median (range)	43 (11–364)	67 (21–247)
% elevated (*n*)	70.37% (19)	81.25% (26)
BMI (%)		
Overweight	7.40% (2)	37.50% (12)
Obese	18.52% (5)	6.25% (2)
Hepatitis		
Minimal (*n*)	0% (0)	3.12% (1)
Mild (*n*)	14.81% (4)	15.63% (5)
Moderate (*n*)	74.08% (20)	53.13% (17)
Severe (*n*)	11.11% (3)	28.12% (9)
Steatosis		
Absent (*n*)	33.33% (9)	37.50% (12)
Minimal (*n*)	40.74% (11)	31.25% (10)
Moderate (*n*)	14.82% (4)	18.75% (6)
Severe (*n*)	11.11% (3)	12.50% (4)
Fibrosis stage [c]		
F 0–1 (*n*)	33.33% (9)	31.25% (10)
F 2 (*n*)	51.85% (14)	31.25% (10)
F 3 (*n*)	14.82% (4)	31.25% (10)
F 4 (*n*)	0% (0)	6.25% (2)
Lymphoid follicle		
% patients (*n*)	40.74% (11)	71.87% (23)
Bile duct damage		
% patients (*n*)	77.78% (21)	100% (32)

[a]Data available only from 16 patients.
[b]Difference in duration of infection between paediatric and adult cases was statistically significant ($p < 0.0001$). ND, not determined; ALT, alanine aminotransferase. Normal ALT levels for paediatric and adult patients were ≤32 and ≤40, respectively, when test was done at 37°C. BMI, body mass index.
[c]Fibrosis according to METAVIR.

Hepatic detection of HCV

Infected hepatocytes were evidenced by detecting viral NS3 protein by manual immunohistochemistry (IHC). Epitope retrieval was performed with sodium citrate buffer (0.01 M, pH 6) in an autoclave for 3 min (20 psi). Endogenous biotin was blocked with Biotin Blocking System (Avidin/Biotin Blocking Kit, Vector Laboratories Inc, Burlingame, USA). A primary mouse antibody to NS3 (1:25, clone MMM33, Abcam, Cambridge, UK) was used for 1 h at 25°C and staining was obtained by applying the streptavidin-biotin peroxidase (SBP) system and substrate-chromogen reagent (Vectastain Elite ABC and DAB Substrate Kit for Peroxidase, Vector Laboratories Inc, Burlingame, USA). Positive staining was not observed without the NS3 primary antibody or with isotype control. No labelling was detected when evaluating antibody specificity on chronic HBV-infected liver samples.

Apoptotic hepatocytes

Activated caspase-3 (casp-3a) labelling was performed using rabbit polyclonal anti-casp-3a antibody (1:1000, AF835, R&D systems, Minneapolis, MN, USA) as previously described [29]. Non-alcoholic steatohepatitis liver biopsy samples that were casp-3a positive were used as positive control. Isotype control was performed.

DNA fragmentation was visualized using the ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (CHEMICON International, Inc., Billerica, MA, USA) according to the manufacturer's instructions. For counterstaining and mounting, DAPI in PBS-glycerol (DAPI/antifade Solution, CHEMICON International, Inc.) was used.

Immunohistochemical detection of $CD4^+$, $CD8^+$, $Foxp3^+$ and $CD20^+$ cells in P-P/I infiltrate

Characterization of the P-P/I infiltrate was performed using monoclonal antibodies: mouse anti-human CD4 (clone 1F6, Novocastra, Newcastle, UK), mouse anti-human CD8 (clone C8/144B, DakoCytomation, Carpinteria, CA, USA), mouse anti--Foxp3 (10 mg/mL, clone 236A/E7, Abcam, Cambridge, UK) and mouse anti-CD20 (clone L26, Novocastra).

After epitope retrieval [sodium citrate buffer (0.01 M, pH 6) in autoclave during 5 min (20 psi)], sections were incubated with each primary antibody and stained by applying the PolyTek HRP anti-Mouse Polymerized Imaging System (ScyTek Laboratories, Logan, UT, USA) according to the manufacturer's instructions. Tonsil sections were used as positive controls and isotype controls were performed.

Quantification

Immunostained and total hepatocytes were counted in 20 high-power fields (1000×). To characterize P-P/I infiltrates, immunostained and total P-P/I lymphocytes were counted in 10 portal tracts (400×).

Double immunostaining

The casp-3a/TUNEL: the TUNEL protocol was carried out according to the manufacturer's instructions. Tissue pretreatment was performed with Proteinase K (20 mg/mL) for 15 min at 25°C. After the anti-Digoxigenin conjugate step, sections were incubated in darkness with anti-casp-3a, followed by a secondary biotinylated antibody (LSAB+System-HRP, DakoCytomation) and streptavidin-AlexaFluor® 546 conjugate (Invitrogen Molecular Probes, Eugene, OR, USA). Finally, samples were counterstained and mounted with DAPI/antifade solution.

$NS3^+$/$TUNEL^+$: NS3 detection was carried out as described above and after chromogen addition the TUNEL protocol was performed.

CD4/Foxp3 and CD8/Foxp3: either CD4 or CD8 immunodetection was performed as described above. After signal amplification, a blocking step was carried out using PBS 1% bovine serum albumin (30 min at 25°C). Subsequently, sections were incubated for 1 h at 25°C with anti-Foxp3 antibody. Detection was performed using an anti-mouse antibody conjugated to FITC (1:100, JacksonImmuno Research, West Grove, PA, USA) for 18 h at 4°C. Finally, samples were counterstained and mounted with DAPI/antifade solution. Isotype controls were also performed.

As detection of NS3, CD4 and CD8 antigens was done using an SBP system and DAB as substrate, images were converted to a false red colour with the Image-Pro Plus software version 6.0.0.260 and then merged with fluorescent ones.

Statistical analysis

Statistical analysis was performed using GraphPad InStat software, version 3.05 (Graphpad, San Diego, CA, USA). The Mann–Whitney *U*-test and unpaired *t*-test were used to compare sets of data. Pearson's and Spearman's correlations were applied. $p < 0.05$ values were considered significant.

Results

HCV NS3 detection in liver biopsies

A cytoplasmic, mainly perinuclear, NS3 immunostaining with a granular pattern of variable intensity was observed (Fig. 1a,b). In both patient groups, infected hepatocytes were not uniformly distributed; in some cases they were observed in intralobular and periportal areas while in others they were observed only in periportal areas.

Even though the number of infected hepatocytes was variable, it was significantly increased in paediatric samples (paediatrics vs. adults median (range): 0.210 (0.004–0.880) vs. 0.105 (0.006–0.449), p 0.0078). In 59.26% of paediatric biopsies, the frequency of infected hepatocytes was higher than 20%, whereas in 73.68% of adult ones it was lower than 20%.

No significant correlation with viral load, serum aminotransferases or hepatitis severity was found; however, an association with fibrosis severity ($p = 0.03$) was observed only in children (Fig. 1).

Apoptosis in liver tissue sections

All typical signs of apoptosis are the final result of a complex biochemical cascade of events. Apoptotic signalling mainly converges in the activation of intracellular caspases, a family of cystein-dependent aspartate-directed proteases that cleave key cellular proteins [30]. 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]. Given these facts, it is recommended that apoptosis should be assessed by multiple methodologies. In consequence, in this study, the activation of caspase-3 and DNA fragmentation, the final and irreversible event in the process of apoptosis, were evaluated.

On H&E-stained sections, non-apoptotic cells were identified by their morphology; however, apoptosis marker detection allowed for the identification of a significant number of apoptotic cells without a specific location pattern (Fig. 2 Panel 1 a-c). The inclusive analysis of apoptosis markers showed a significant correlation between casp-3a$^+$ and TUNEL$^+$ hepatocytes (in children $r = 0.595$, p 0.002; in adults $r = 0.558$, p 0.003). The timing of the apoptotic process events was evidenced through double immunostaining because an excess of casp-3a$^+$ over TUNEL$^+$ hepatocytes as well as an increased casp-3a labelling intensity in double-positive hepatocytes was observed (Fig. 2, Panel 2 a-d). On the other hand, apoptotic hepatocyte number was significantly higher in CHC samples compared with control ones (Table 2). Meanwhile, casp-3a quantification in adult CHC samples was higher than in paediatric samples ($p = 0.0376$), and no differences were observed between control samples from both groups (Table 2). In paediatric biopsies, HCV$^+$ hepatocytes correlated with casp-3a$^+$ ($r = 0.748$, $p < 0.0001$) and TUNEL$^+$ cells ($r = 0.606$, $p = 0.0017$). Moreover, the number of infected hepatocytes exceeded that of apoptotic cells in nearly all cases (100% casp-3a$^+$; 92.59% TUNEL$^+$). Given the observed correlations, double immunostaining was performed in both age groups. Due to the technical incompatibility of antigen exposure procedures, this approach could only be achieved for NS3 and TUNEL. As shown in Fig. 2 (Panel 3 a-d), induction of apoptosis was detected in most infected hepatocytes from paediatric patients. In contrast, no correlation between apoptosis and infected hepatocytes was observed in adult samples.

Regarding liver damage, although TUNEL staining showed no significant association, casp-3a$^+$ hepatocytes count was associated with fibrosis stage (p 0.026) in paediatric biopsies (Fig. 3a) and with severe hepatitis (p 0.009) in adult biopsies (Fig. 3d).

Detection of CD4$^+$, CD8$^+$, Foxp3$^+$ and CD20$^+$ cells in P-P/I infiltrate

CD4$^+$, CD8$^+$, Foxp3$^+$ and CD20$^+$ cells were predominantly identified in the P-P/I areas (Fig. 4, Panel 1 a-d) while only scattered lymphocytes were observed in the intralobular component. The total numbers of P-P/I infiltrating lymphocytes as well as CD4$^+$, CD8$^+$ and Foxp3$^+$ cells were higher in adult samples than in paediatric ones [paediatrics vs. adults median

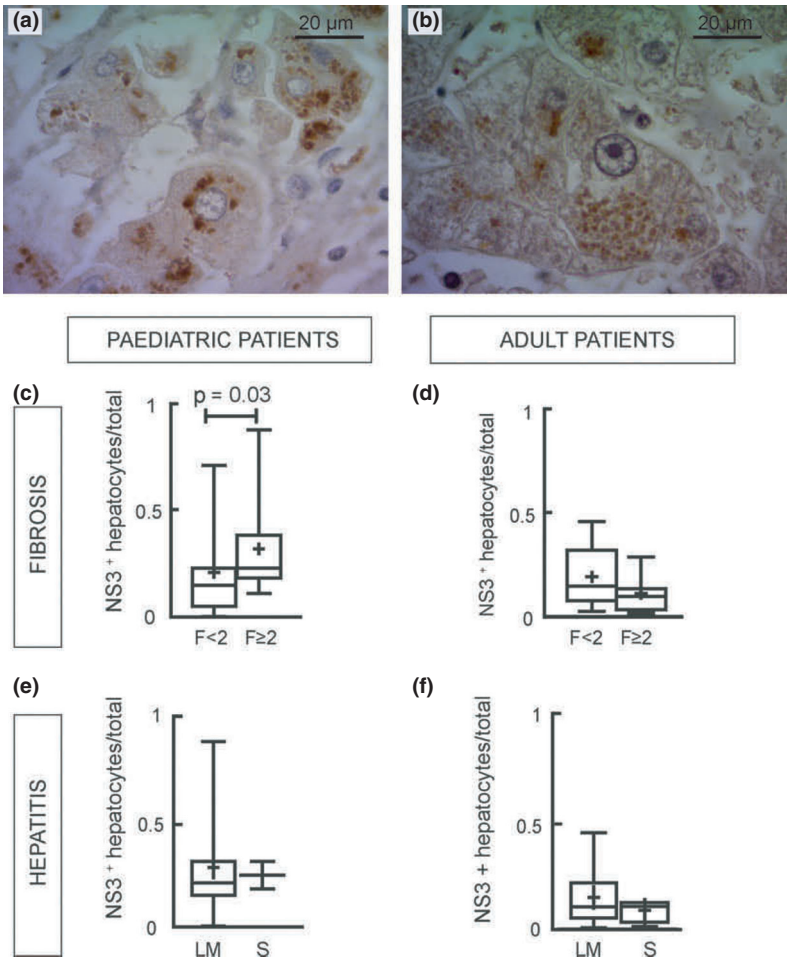

FIG. 1. Infected hepatocytes in liver biopsies of CHC patients. NS3 immunostaining (a, b). Infected hepatocytes showing NS3 cytoplasmic granules. Infected hepatocytes related to fibrosis (c, d). Significant fibrosis ($F \geq 2$) according to METAVIR. Infected hepatocytes related to hepatitis (e, f). L, mild; M, moderate; S, severe hepatitis. Paediatric patients (c, e) and adult patients (d, f). The results are depicted in box plots. Horizontal lines within boxes indicate medians. Horizontal lines outside the boxes represent the 5 and 95 percentiles. Mean is indicated as +. Labelled hepatocytes/total hepatocytes in 20 high-power fields (1000×). The Mann-Whitney *U*-test and unpaired *t*-test were used to compare sets of data.

(range): total P-P/I lymphocytes, 80 (20–580) vs. 373 (198–906), p < 0.0001; $CD4^+$ cells, 0.031 (0–0.402) vs. 0.138 (0.017–0.503), p 0.022; $CD8^+$ cells, 0.314 (0.040–0.710) vs. 0.485 (0.247–0.642), p 0.0115; $Foxp3^+$ cells, 0.035 (0–0.167) vs. 0.146 (0.031–0.318), p < 0.0001; $CD20^+$ cells, 0.300 (0.040–0.610) vs. 0.350 (0.055–0.660), p 0.46].

In both age groups CD4/Foxp3, CD8/Foxp3 and CD20/Foxp3 ratios were calculated for each patient [in children, median (range): CD4/Foxp3, 2.16 (0–11.57); CD8/Foxp3, 6.25 (1.44–30.18); CD20/Foxp3, 5.08 (1.69–22.57); in adults: CD4/Foxp3, 0.979 (0.06–3.44); CD8/Foxp3, 3.23 (1.18–10.89); CD20/Foxp3, 2.39 (1.05–4.59)] to evaluate the proportion of each cell type when compared with $Foxp3^+$ cells. In the adult cases, according to CD4/Foxp3 median, it could be assumed that the $CD4^+$ cell population was mainly composed of $Foxp3^+$ cells; hence, CD4/Foxp3 double immunostaining was performed to validate this. Moreover, as *in vitro* studies showed $CD8^+/Foxp3^+$ cells with regulatory activity [31], CD8/Foxp3 double immunostaining was also performed. In P-P/I infiltrates $CD4^+/Foxp3^+$ cells were identified in both groups, with a higher frequency in adult samples (Fig. 4, Panel 2 a-e), while no $CD8^+/Foxp3^+$ cells were present at all (Fig. 4, Panel 2 f-j).

In paediatric patients, $CD8^+$ cells correlated with both infected ($r = 0.495$, p = 0.04) and $TUNEL^+$ hepatocytes ($r = 0.474$, p 0.047). In adult cases correlations between $CD4^+$ and $CD8^+$ cells with casp-$3a^+$ hepatocytes ($CD4^+$, $r = 0.624$, p 0.0005; $CD8^+$,$r = 0.387$, p 0.04) were observed.

Regarding histological parameters, none of the lymphocyte populations studied was associated with liver damage in children. Only inflammatory activity correlated with total P-P/I lymphocyte count ($r = 0.694$, p 0.0002). In adult patients, no differences were found regarding fibrosis severity; instead, $CD8^+$ cell count was associated with hepatitis severity (p < 0.0001)(Fig. 4, Panel 3 g) and also correlated with inflammatory activity ($r = 0.639$, p 0.0003).

Discussion

HCV pathogenesis is a very complex phenomenon that still requires further investigation in order to determine the actual

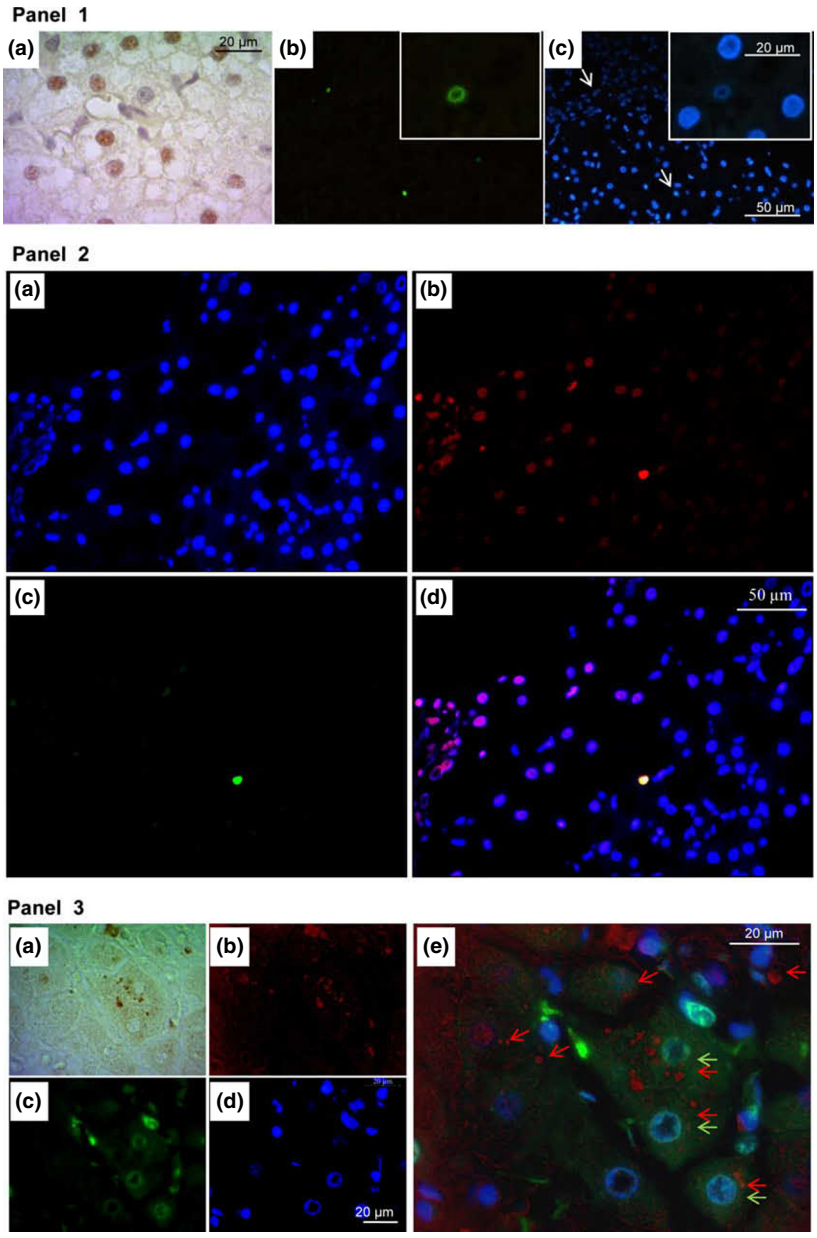

FIG. 2. Panel 1: Apoptosis markers in liver biopsies of CHC patients. Immunostaining of liver biopsies. (a) Casp-3a$^+$ cells displaying nuclear staining; (b) fluorescent nucleus in TUNEL$^+$ cells; (c) DAPI counterstaining. Panel 2: Double immunostaining casp-3a/TUNEL. (a) DAPI counterstaining; (b) Casp-3a$^+$ hepatocytes; (c) TUNEL$^+$ hepatocyte; (d) merged images.Panel 3: NS3/TUNEL double immunostaining. (a) NS3; (b) false red colour; (c) TUNEL; (d) DAPI counterstaining; (e) merged images. Note that the number of infected hepatocytes exceeded that of apoptotic ones in liver samples from paediatric patients. Red and green arrows indicate infected and apoptotic hepatocytes, respectively.

role of each of its contributing factors. To date, given the difficulty in obtaining HCV-infected liver tissue biopsies, pathogenesis has mainly been studied in *in vitro* and *in vivo* models. Considering that apoptosis and immune response play an important role in the process and that little is known about CHC in children, the focus of our analysis was to determine the potential role and contribution of HCV, apoptosis and immunity in the pathogenesis of CHC in both adults and children.

HCV infection is a dynamic process and only limited data are available about the proportion of infected hepatocytes during disease. In our series, we reported that the total amount of HCV infected hepatocytes in most adult cases was less than 20%, while it was significantly higher in children. It is accepted that HCV spreading among hepatocytes is controlled by the intrahepatic immune response during chronic infection. In our paediatric sample series, we observed an excess of HCV$^+$ hepatocytes, which implies that the immune system could not completely achieve virus control. In contrast, in our adult cases immune response is more represented, as it will be discussed later. A role for the immune response was also suggested by other authors who detected 50–70% infected hepatocytes in liver biopsies from adults with acute hepatitis C infection while only nearly 20% of infected hepatocytes were

TABLE 2. Quantification of apoptosis markers in control and CHC liver samples

	Casp-3a$^+$hepatocytes[a]		TUNEL$^+$hepatocytes[a]	
	Median (range)	p value	Median (range)	p value
Paediatric samples				
Controls	0.001 (0–0.005)	<0.0001	0.000 (0–0.003)	0.0003
CHC	0.064 (0–0.287)		0.057 (0–0.311)	
Adult samples				
Controls	0.001 (0–0.009)	<0.0001	0.001 (0–0.003)	0.0005
CHC	0.091 (0–0.324)		0.057 (0–0.311)	
Paediatric vs. adult controls		0.1889		0.1358
Paediatric vs. adult CHC		0.0376		0.35

[a]Number of positive hepatocytes/number of total hepatocytes (1000×).

detected in chronic infection samples [32,33]. Furthermore, in our series, infected hepatocytes seemed to contribute to fibrosis development exclusively in children. In contrast, in our adult cases, the presence of virus was not correlated with liver damage, as has been previously proposed by Rodriguez-Inigo *et al.* [34]. Finally, no correlation was observed between the number of infected hepatocytes and viral load in either age groups, a fact that has also been previously described [32, 35, 36]. A plausible explanation for this observation is that the IHC approach exclusively detects viral antigens but does not take into account the actual number of viral particles or their input into the plasma compartment.

On the other hand, concerning apoptosis, we demonstrated that this process is a prominent liver feature in CHC patients because a significantly higher number of apoptotic cells was identified in CHC biopsies compared with controls, another fact that is in agreement with previous reports [9, 10, 37–43]. Regarding the role of apoptosis in pathogenesis, many studies including adult CHC cohorts described conflicting results [10, 37, 39–41, 43, 44]. In our series, when comparing both age groups, contrasting outcomes were observed. In children, apoptosis markers were related to infected hepatocytes and fibrosis severity. Furthermore, apoptosis was detected as an almost exclusive event of infected hepatocytes by double immunostaining. The scenario was different in adults: as previously reported [10, 44, 45], there was a high predominance of apoptotic non-infected hepatocytes along with an association of apoptosis with hepatitis severity. As apoptosis, unlike necrosis, occurs without releasing intracellular components and does not result in an inflammatory process [46], apoptosis could be considered the result of the inflammation rather than its cause.

The liver is a mediator of local and systemic immunity and hence, an important immune regulation site [47]. It is considered that HCV persistence is the consequence of multiple viral factors that interfere with immunity. Depending on the severity of dysfunction, the HCV-specific immune response may successfully control viral infection or fail to do so, while allowing the infiltrating cells to persist in the liver and contribute to fibrosis [4, 45].

The assessment of the role played by the immune system in the pathogenesis of CHC in the studied adult cohort showed that CD8$^+$ cells were increased in severe hepatitis

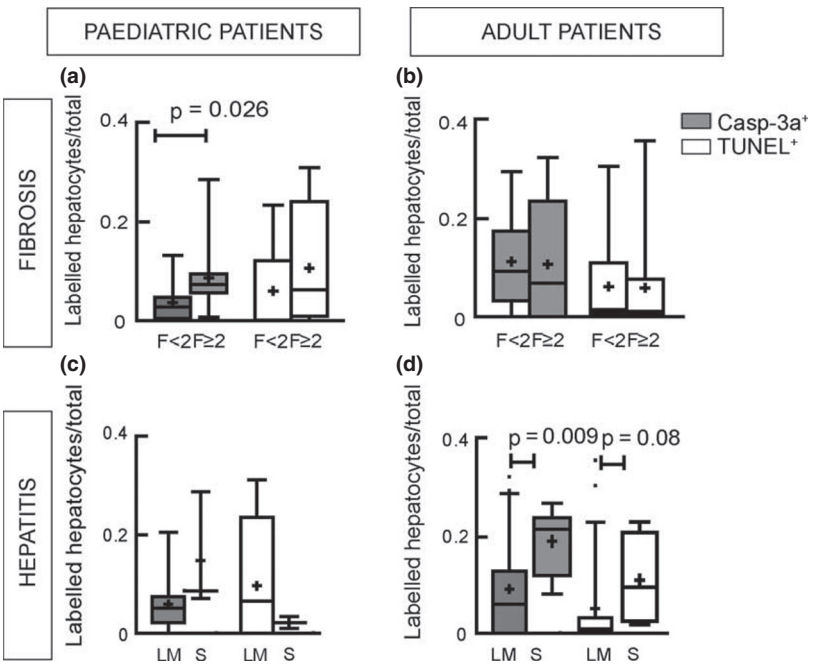

FIG. 3. Apoptosis markers related to fibrosis (a, b) and to hepatitis (c, d). Significant fibrosis ($F \geq 2$) according to METAVIR. L, mild; M, moderate; S, severe hepatitis. Paediatric patients (a, c) and adult patients (b, d).The results are depicted in box plots. Horizontal lines within boxes indicate medians. Horizontal lines outside the boxes represent the 5 and 95 percentiles. Mean is indicated as +. Labelled hepatocytes/total hepatocytes in 20 high-power fields (1000×). The Mann-Whitney *U*-test and unpaired *t*-test were used to compare sets of data.

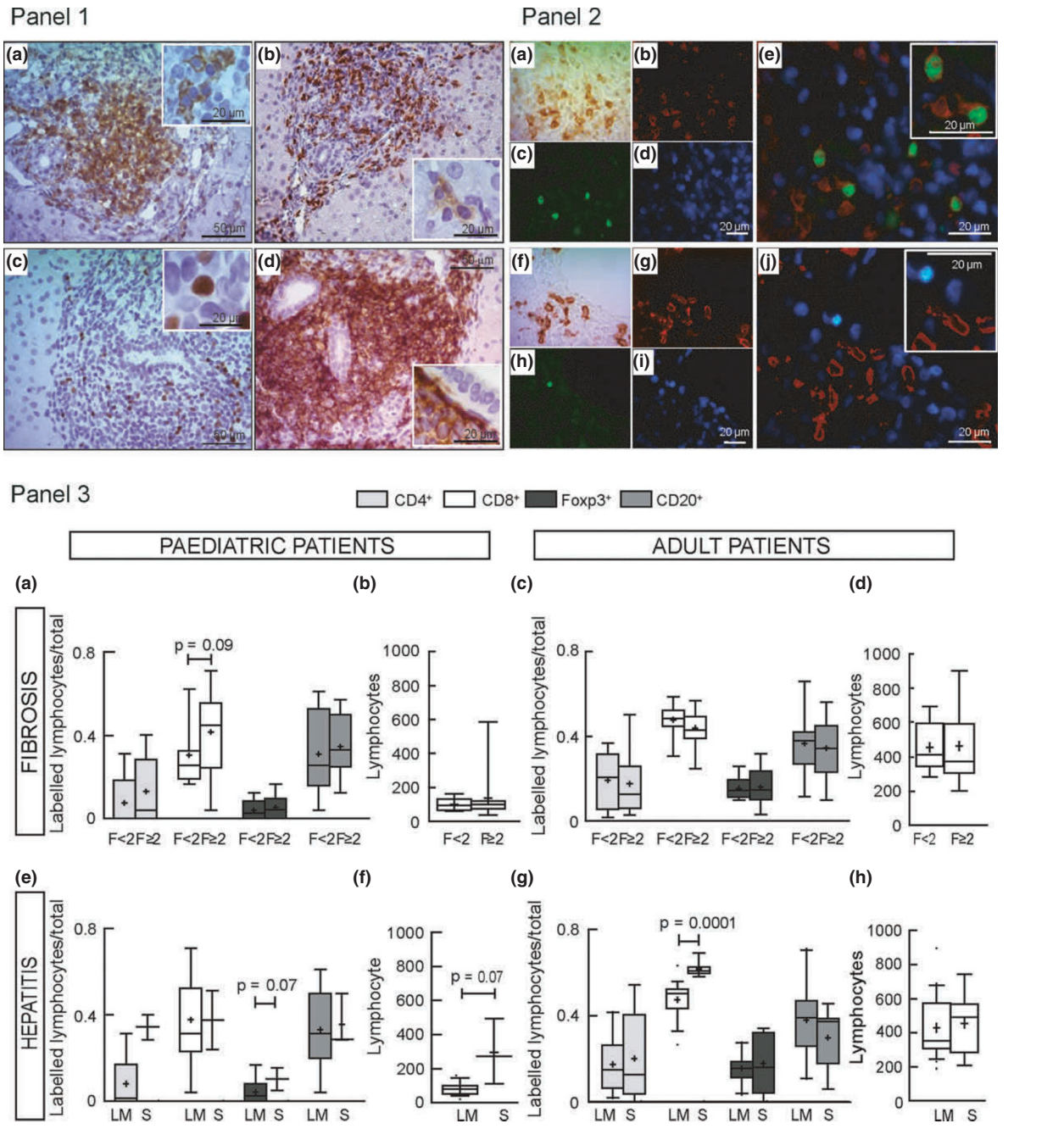

FIG. 4. Panel 1: $CD4^+$, $CD8^+$, $Foxp3^+$ and $CD20^+$immunostaining in liver biopsies of CHC patients. (a) $CD4^+$; (b) $CD8^+$; (c) $Foxp3^+$; (d) $CD20^+$ cells. Panel 2: CD4/Foxp3 and CD8/Foxp3 double immunostaining. (a) $CD4^+$; (b) false red colour; (c) $Foxp3^+$; (d) DAPI counterstaining; (e) merged images (the insertion shows double positive cell amplification);(f) $CD8^+$; (g) false red colour; (h) $Foxp3^+$; (i) DAPI counterstaining;(j) merged images (the insertion shows amplification). Panel 3: $CD4^+$, $CD8^+$, $Foxp3^+$, $CD20^+$and total P-P/I lymphocytes related to fibrosis (a-d) and to hepatits (e-h). $CD4^+$, $CD8^+$, $Foxp3^+$ and $CD20^+$ (a, c, e, g) and total P-P/I lymphocytes (b, d, f, h). Paediatric patients (a, b, e, f) and adult patients (c, d, g, h). Significant fibrosis ($F \geq 2$) according to METAVIR. L, mild; M, moderate; S, severe hepatitis. The results are depicted in box plots. Horizontal lines within boxes indicate medians. Horizontal lines outside the boxes represent the 5 and 95 percentiles. Mean is indicated as +. (Labelled P-P/I lymphocytes)/(total P-P/I lymphocytes) in 10 portal tracts (400×). The Mann-Whitney *U*-test and unpaired *t*-test were used to compare sets of data.

and correlated with inflammatory activity, but there was no association with fibrosis severity. Besides, $CD4^+$ and $CD8^+$ cell count correlated with apoptotic hepatocytes. As we have just demonstrated, only a small fraction of hepatocytes were infected in adult biopsies, but still, many were apoptotic, so it could be hypothesized that apoptosis activation is not directly triggered by HCV, but instead could be due to the antiviral immune response. Therefore, non-infected hepatocytes could represent a major contribution to the overall liver injury. In fact, it has been reported that HCV-specific $CD8^+$ cells represent only a fraction of the whole intrahepatic $CD8^+$ cell population [18]. In this sense, our results support the 'Bystander killing' theory [45, 48, 49], which suggests that HCV-specific cytotoxic cells eliminate infected hepatocytes by apoptosis induction and the release of soluble effectors. Although these soluble effectors aid in the control of the infection, they also attract non HCV-specific lymphocytes, which in turn affect non-infected hepatocytes. In contrast, in the paediatric cohort, the total P-P/I lymphocyte numbers were lower, and this may reflect a lighter immune impact on liver damage. Nevertheless, the observed relationship among infected hepatocytes, $CD8^+$ cells and apoptosis may mirror the classic immune response to viral infection in which cytotoxic lymphocytes attempt to eliminate infected cells.

With regards to $Foxp3^+$ cells, we demonstrated by simple and double immunostaining approaches that $Foxp3^+$ cells constitute a large lymphocyte fraction in CHC biopsies, which are mainly located in areas where $CD8^+$ cells are predominant. This observation is also in accordance with previous reports [24, 50–52]. Moreover, although $CD8^+$/$Foxp3^+$ cells have been described in CHC biopsies [31, 53],they were under-represented in our studied series, as in other reports [51,52]. On the other hand, in our series $Foxp3^+$ cells did not have a clear impact on fibrosis in either of the studied cohorts because the proportion of $Foxp3^+$ cells remained similar in both mild and severe fibrosis, which is in agreement with other authors [26, 51, 52]. It is well known that Treg cells participate in a delicate intrahepatic balance that results in the attenuation of protective immunity and immune-mediated damage [50]; however, concerning their role in CHC liver injury, several controversies exist, particularly related to fibrosis [50–52, 54, 55]. Nevertheless, it should be considered that the expression of Foxp3 is not equivalent to differentiation of T cells as regulatory T cells, because up-regulated Foxp3-expression has also been observed during T-cell activation [56]. The Treg population in HCV-infected livers is heterogeneous, and both phenotypical as well as functional features must be considered in future studies to finally characterize their role in the pathogenesis [26, 50–52, 57].

HCV, apoptosis and the immune response are all involved in CHC pathogenesis in both age groups, although certain differences regarding the weight of each component in the final scenario were observed between children and adults. Based on these results, we propose a model of how HCV pathogenesis could be developed in each age group (Fig. S1). In paediatric patients, it would be largely HCV that participates in liver damage. HCV would not have a direct effect on histological parameters, but instead it would participate through apoptosis induction, which in turn contributes to fibrosis development. In contrast, in adult patients, the prevalence of apoptotic non-infected hepatocytes, related to hepatitis severity, may indicate that apoptosis could be mainly a consequence of the immune response to HCV. It could be hypothesized that the lower impact of the immune response in children may be related to the high proportion of vertically infected cases included. As immune system maturation would be concomitant with HCV infection, this could result in a tolerogenic environment and consequently the observed damage could largely mirror a viral effect. In an opposite manner, it could be assumed that in adults the virus has been challenging the immune system for a long time, leading to a major role of the immune response in the observed damage. In line with this, we previously described the phenomenon of immune tolerance when we evaluated the evolution of HCV hypervariable region 1 (HVR1) in immunocompetent children born to HCV-infected mothers [58]. The lack of HVR1 variability observed in the studied paediatric cases may reflect the adaptation of the virus to a particularly tolerant immune environment. Moreover, in a longer follow-up study including those same paediatric patients, we observed in one case at month 115 a switch of quasi species community after 60 months of an almost completely invariable HVR1 amino acid sequence. Furthermore, the quasis peciess witch correlated with the abrupt worsening of the patient's clinical status [59]. Based on these results we performed the pathogenesis analysis on liver biopsy samples of this particular case during the follow-up period. The present analysis revealed a decrease in the infected hepatocyte number concomitant with an increase of apoptotic cells, $CD4^+$, $CD8^+$, $Foxp3^+$ and total P-P/I lymphocyte number (Fig. S2). This last scenario observed in a grown-up paediatric patient mirrors the one in adults.

Of course, differences between age groups such as patient's ages, infection acquisition age, duration of infection, immune system characteristics according to age, sex and other cofactors cannot be omitted when analysing the obtained results. We are aware that it is the major limitation of our study but, due to the incidence of CHC and the type of sample evaluated here, standardization of these variables is almost impossible, particularly in children. Even though the number of

paediatric cases with severe liver damage may seem low, the slow progression of liver injury renders this stage quite uncommon in children.

Finally, this report represents a novel study that evaluates intrahepatic infection, apoptosis and infiltrating lymphocytes in liver samples to finally characterize their involvement in CHC pathogenesis; these are aspects that, to the best of our knowledge, have never before been thoroughly evaluated in children. Although this study could be considered as a single-centre descriptive analysis, it may lay down the groundwork and lead to a larger multicentre cohort study that could finally validate these findings.

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Transparency Declaration

The authors disclose no financial conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic pathogenesis mechanism proposed: HCV, apoptosis and immune response proved to be involved in CHC pathogenesis of both paediatric and adult patients; however, certain differences regarding the role that each component plays in the final scenario were observed between children and adults. In paediatric patients, it would be largely HCV that participates in liver damage. HCV would not have a direct effect on histological parameters, but instead it would participate through apoptosis induction, which in turn contributes to fibrosis development. In contrast, in adults pathogenesis would be mainly associated with an exacerbated immune response; furthermore, apoptosis could be a consequence of the immune response to HCV.

Figure S2. Evaluation of one CHC paediatric case during follow-up. Immunohistochemical detection of $NS3^+$ and casp-$3a^+$ hepatocytes as well as $CD4^+$, $CD8^+$, $Foxp3^+$, $CD20^+$ and total P-P/I lymphocytes in liver biopsies. Patient (male, maternal HCV infection risk factor) who showed a rapid progression of injury, requiring multiple biopsies during the follow-up and resulting in severe hepatitis and incomplete cirrhosis. Quantification: Labelled hepatocytes/total hepatocytes in 20 high-power fields (1000×) for NS3 and casp-3a immunostaining; (Labelled P-P/I lymphocytes)/(total P-P/I lymphocytes) in 10 portal tracts (400×) for CD4, CD8, Foxp3 and CD20 immunostaining. Age at biopsy and histological features of liver samples are shown under the graph. Fibrosis according to METAVIR.

Tabla S1. Clinical, virological and histological features of each HCV paediatric patient.

Table S2. Clinical, virological and histological features of each HCV adult patient.

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