INCREASED HEPATIC EXPRESSION OF TOLL-LIKE RECEPTORS (TLR) 2 AND 4 IN THE HEPATIC INFLAMMATION-FIBROSIS-CARCINOMA (IFC) SEQUENCE

Running title: TLR2 and 4 in hepatic IFC sequence

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ABSTRACT

We aimed to evaluate TLR2 and TLR4 expression in combination with proinflammatory genes (NF-κB, TNF-α and COX-2) in liver samples of patients in different stages of liver disease. Fifteen patients with unexplained transaminases elevation (control group), 22 with viral chronic hepatitis (hepatitis group), 14 with virus-induced severe fibrosis/cirrhosis (cirrhosis group) and 10 with hepatocarcinoma (hepatocarcinoma group), who underwent liver biopsy or surgical intervention, were consecutively included in the study. Quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2 mRNA was done by real-time RT-PCR and TLR2 and TLR4 protein expression was evaluated by immunohistochemistry. As compared with control, we found an increased TLR2 and TLR4 mRNA expression in hepatitis (TLR2:2.66±0.69, p=0.04; TLR4:3.11±0.79, p=0.03) and cirrhosis (TLR2:2.14±0.5, p=0.04; TLR4:1.74±0.27, p=0.008). This was associated with an increased TNF-α and COX-2 mRNA expression in hepatitis (TNF-α:3.24±0.79, p=0.02; COX-2:2.47±0.36, p=0.003) and cirrhosis (TNF-α:1.73±0.28, p=0.009; COX-2:1.8±0.35, p=0.04), whereas NF-κB mRNA was increased in hepatitis (2.42±0.31, p=0.0003) but maintained in cirrhosis (1.34±0.17, p=0.3). Immunohistochemistry confirmed increased protein expression of TLR2 and TLR4 in hepatitis and cirrhosis and a maintained expression of these receptors in hepatocarcinoma. Upregulation of TLR2, TLR4 and their proinflammatory mediators is associated with human hepatic IFC sequence induced by viral chronic hepatitis.

Word count: 198

Keywords: chronic hepatitis, cirrhosis, hepatocarcinoma, TLR2, TLR4

INTRODUCTION

Chronic liver inflammation, irrespective of the underlying cause (metabolic, immune-driven, or virus-induced), leads to fibrosis/cirrhosis which are a precancerous state in which the development of hepatocarcinoma is more likely. Some authors call this sequence the hepatic inflammation-fibrosis-carcinoma (IFC) sequence [1]. Nevertheless, the cellular and molecular effectors mediating the interplay between the components of hepatic IFC sequence continue largely unknown.

Recently, several studies implicated Toll-like receptors (TLRs) as potential key orchestrators of the hepatic IFC sequence [2-5]. TLRs are one of the most representative classes of pathogen–associated molecular patterns (PAMPs) receptors that play a critical role in innate immunity activation [6-7]. The human TLR family consists of ten members that enable the innate immunity system to recognize different groups of pathogens while initiating appropriate and distinct immunological responses, according to the recognized PAMP [6-7]. Besides immune cells, most liver cells (hepatocytes, Kupffer cells and stellate cells) also express TLRs and respond to their ligands [8-9]. TLR2 and TLR4 have been the most studied TLRs in liver diseases as they sense bacterial components and may thus mediate liver injury associated with increased bacterial translocation that is present in many liver diseases [10-12]. TLR2 is essential for the innate immune response to Gram-positive bacteria, being activated by bacterial lipoproteins and peptidoglycan [6-7]. TLR4 acts as a receptor for lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria [6-7]. Besides exogenous ligands, TLR2 and TLR4 may also sense endogenous ligands initiating danger signals, such as high mobility group box 1, hyaluronan and heat shock protein 60, inducing an inflammatory response in the absence of microbial challenge [13]. Stimulation of these two receptors initiates a signaling cascade that promotes activation of nuclear factor (NF)-κB and mitogen activated protein kinases (MAPK) and consequently production of different pro-inflammatory mediators such as tumour necrosis factor alpha (TNF-α) and cyclooxygenase-2 (Cox-2) [14-18].

Recent animal studies and *in vitro* hepatocyte culture models suggest that TLR2 and TLR4 may play a key role in the hepatic IFC sequence. Modulation of TLR2 and/or TLR4 function was shown to influence liver inflammation in chronic liver diseases such as alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), chronic hepatitis C and chronic hepatitis B [2-3].

There is also accumulating evidence that TLR4-induced activation and sensitization of hepatic stellate cells (HSCs) may constitute an important molecular link between hepatic inflammation and fibrogenesis [19-22]. Moreover, a recent study has revealed TLRs, in particular TLR4, as major factors linking hepatic chronic inflammation and hepatocarcinoma [23].

However, to date, the suggested implication of TLR2 and TLR4 in the pathogenesis of hepatic IFC sequence is principally based on evidence obtained from animal studies or in vitro hepatocyte culture models. Studies using diseased human liver tissue to confirm or refute the *in vitro* and animal findings are scarce and have evaluated TLR2 and TLR4 in each stage of IFC sequence separately.

Therefore, in the present study, we evaluated the expression of TLR2 and TLR4 in liver samples from patients in each stage of virus-induced hepatic IFC sequence. The expression of NF-κB, TNF-α and COX-2 was also evaluated in order to characterize their association with TLR2 and TLR4 expression.

MATERIAL AND METHODS

Patients and biological samples

This study included patients from two hospitals of the North of Portugal (Braga Hospital and Portuguese Oncology Institute of Porto). The study protocol respected the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Braga Hospital and Portuguese Oncology Institute of Porto. Informed consent was obtained from each patient.

Patients were recruited consecutively during 2009. We defined four groups: control, hepatitis, cirrhosis and hepatocarcinoma. Control group included patients followed in the Hepatology outpatient clinic of Braga Hospital who underwent liver biopsy because of chronic unexplained transaminases elevation. We excluded from this group patients with alcohol abuse (>30g/d in males; >20 g/d in females), analytical or histological findings favoring hemocromatosis, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, HIV infection or clinical, analytical, imagiological or histological evidence of severe fibrosis/cirrhosis (METAVIR F3-4). Hepatitis group included chronic hepatitis B or C patients followed in the Hepatology outpatient clinic of Braga Hospital who underwent staging liver biopsy. Cirrhosis group was selected from the same group of patients but with histological evidence of severe fibrosis/cirrhosis (METAVIR F3-4). Hepatocarcinoma group included chronic hepatitis B or C patients with diagnosis of hepatocarcinoma (according to the EASL 2000 Barcelona Guidelines [24]) followed in the outpatient clinic of Portuguese Oncology Institute of Porto who underwent surgical resection of hepatocarcinoma. In these groups, patients must had >18 years, serological evidence of chronic hepatitis B (HBsAg+) or C (HCVAb+) and clinical stability. Histological evidence of cirrhosis and hepatocarcinoma was required in the hepatocarcinoma group. Patients with HIV infection or analytical or histological findings suggestive of liver disease other than viral chronic hepatitis were excluded.

Before liver biopsy or surgical intervention, blood samples were drawn from fasting patients for routine analysis (complete blood count, glucose, electrolytes, renal and liver function tests and

coagulation study) and viral load quantification. Liver tissue was obtained by percutaneous biopsy using a 16-gauge Menghini needle or by transjugular biopsy. Hepatocarcinoma tissue (for immunohistochemical evaluation of TLR2 and TLR4) and adjacent liver tissue (for cirrhosis confirmation) were obtained from surgical specimen in the hepatocarcinoma group. The collected tissue was divided in two fragments: one was immediately placed in RNAlater (Ambion) and stored at -80ºC for mRNA isolation and quantification; the other was fixed in 10% buffered formalin and embedded in paraffin for histological and immunhistochemical analyses.

mRNA isolation and quantification of TLR2, TLR4, NF-kB, TNF-α and COX-2

Total mRNA was extracted from tissue samples using the TriPure isolation reagent according to the manufacturer's instructions (Roche, Germany). Concentration and purity were assayed by spectrophotometry (Eppendorf 6131000.012). Two-step real-time RT-PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle of graded dilutions from a randomly selected sample from control group. For relative quantification of specific mRNA levels, 100 ng of total mRNA from each sample underwent two-step real-time RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were similar in all experimental groups, which enabled the use of this gene as internal control. RT (20 µL; 10 min at 22ºC, 50 min at 50ºC and 10 min at 95ºC) was performed in a standard thermocycler (Whatman Biometra 050-901). Five percent of the cDNA yield was used as a template for real-time PCR (LightCycler II, Roche) using SYBR green (Qiagen 204143) according to the manufacturer's instructions. Specific PCR primers pairs for the studied genes (GAPDH, TLR2, TLR4, NF-κB, TNF-α and COX-2) are presented in table 1. Results of mRNA quantification were expressed as an arbitrary unit (AU) set as the average value of control group, after normalization for GAPDH.

Immunohistochemical evaluation of TLR2 and TLR4

Tissue specimens were fixed in 10% neutral buffered formalin for 24h and paraffin embedded. Deparaffinized tissue slides were submitted to antigen retrieval using a high temperature antigen unmasking technique in a water bath, 95º in citrate buffer pH6.0, for 20 min. Endogenous peroxidase activity was blocked by incubating the slides with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 min. After washing the slides in distilled water and PBS/0.05% Tween 20 solution, immunostaining was performed using an immunoperoxidase method according to the manufacturer's instructions. The slides were incubated with normal horse serum (Vector Laboratories, Burlingame, CA, USA) 1/50 in PBS-bovine serum albumin (BSA) 1% at room temperature for 20 min in humid chamber. Sections were then incubated with primary antibody at 4ºC overnight. The following primary antibodies were used: rabbit polyclonal antibody anti-TLR2 (H-175, 1:50 dilution, Santa Cruz Biotechnology, California, USA), and rabbit polyclonal anti-TLR4 (H-80, 1:100 dilution, Santa Cruz Biotechnology, California, USA). The slides were then rinsed in PBS/0.05% Tween 20 solution, and bound antibody was detected by applying biotilynated secondary antibody (Vectastain Universal Elite ABC Kit) for 30 min. After wash the slides with PBS/0.05% Tween 20 solution the slides were incubated with ABC reagent (Vectastain Universal Elite ABC Kit) for 30 min. The slides were washed in PBS and incubated for 7 min in 3,3-diaminobenzidine (DAB; Sigma-Aldrich, USA) 0.05g/PBS, 0.03%H_2O_2. Following counterstaining with hematoxylin for 20s, the slides were washed for 4 min in water, dehydrated and mounted with Entellan (Merck KGaA, Darmstadt, Germany). Normal gastric mucosa and lymph node tissue were used as negative and positive controls, respectively. An antibody diluent (non-immune IgG, TA-125-UD; Thermo Scientific) was used in some samples as additional negative control, confirming the specificity of our protocol. We evaluated immunostaining of hepatocytes for TLR2 and TLR4 all the samples. In order to quantify TLRs expression in tissue samples 3 parameters were considered: 1. *Sample positivity*: A sample was considered positive if hepatocytes were clearly marked by the antibody; 2. *Grade of expression*: A score of 0 to 3 was

considered according to the number of epithelial cells marked (0- no cells; 1- less than 10% of epithelial cells; 2- 10-75% cells; 3- more than 75% cells); 3. *Intensity of expression*: A score of 0 to 3 was considered according to a subjective evaluation of the intensity of marked cells (0- no immunostaining; 1- weak positive staining; 2- moderate positive staining; 3- strong positive staining). Immunohistochemical evaluation was performed independently by two experienced pathologists.

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences-SPSS for Windows (version 17.0). Data are presented as mean ± standard error of mean (SEM) or as median and range, according to the type of distribution. Student's t test was used for comparison between groups. When necessary, the test was preceded by a natural logarithm transform to obtain a normal distribution. Correlation between TLR2 and TLR4 mRNA expression and viral load, necroinflammatory activity or transaminases levels was evaluated by univariate analysis. Statistical significance was set at $p<0.05$.

RESULTS

Baseline Characteristics of patients

The baseline characteristics of patients are shown in table 2. A total of 61 patients were included in the study: 15 patients in control group, 22 (10 HBsAg+ and 12 HCVAb+) patients in hepatitis group, 14 (7 HBsAg+ and 7 HCVAb+) patients in cirrhosis group and 10 (4 HBsAg+ and 6 HCVAb+) patients in hepatocarcinoma group. Histological findings in control group included: steatohepatitis (7 patients); macrovesicular steatosis (3 patients); perivenular cholestasis (2 patients); granulomatous hepatitis (2 patients); normal findings (1 patient). Patients with steatohepatitis or macrovesicular steatosis were considered to have non-alcoholic fatty liver disease (NAFLD). As expected, cirrhosis and hepatocarcinoma groups had significantly higher levels of bilirrubin and INR and lower levels of albumin.

mRNA expression of TLR2, TLR4, NF-κB, TNF-α and COX-2

Quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2 mRNA are shown in figure 1. In hepatitis group, expression of TLR2 (2.66 ± 0.69, p=0.04) and TLR4 (3.11 ± 0.79, p=0.03) were greatly increased. This was associated with increased expression of NF-κB (2.42 ± 0.31, p=0.0003), TNF-α (3.24 ± 0.79, p=0.02) and COX-2 (2.47 ± 0.36, p=0.003). Comparing to control group, this increased inflammatory profile (with exception of NF-κB) persisted in cirrhosis group (TLR2: 2.14 ± 0.5, p=0.04; TLR4: 1.74 ± 0.27, p=0.008; NF-κB: 1.34 ± 0.17, p=0.3; TNF-α: 1.73 ± 0.28, p=0.009; COX-2: 1.8 ± 0.35, p=0.04), despite a global but not significant (except for NF-kB) decrease in expression of all the genes when comparing with hepatitis group. There were no differences between chronic hepatitis B or C patients (p>0.05, all genes). We didn´t find any difference or tendency when comparing genetic profile between F0, F1 or F2 patients (p>0.05, all genes). No correlation was seen between mRNA expression of any gene and viral load, necroinflammatory activity or transaminases levels.

Immunohistochemical evaluation of TLR2 and TLR4

All the samples, including those from hepatocarcinoma group, were positive for TLR2 and TLR4. Comparing with control group, TLR2 and TLR4 expression grade was maintained in hepatitis group and reduced in cirrhosis and hepatocarcinoma groups, while TLR2 and TLR4 expression intensity was increased in hepatitis and cirrhosis groups. Moreover, in control group, cytoplasmic staining of hepatocytes for TLR2 and TLR4 was very heterogeneous, while in hepatitis and cirrhosis groups, hepatocytes showed a diffuse cytoplasmic staining for TLR2 and TLR4. Comparing with hepatitis and cirrhosis groups, TLR2 expression grade and intensity and TLR4 expression grade were reduced, while TLR4 expression intensity was maintained in hepatocarcinoma group. In most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining alternating with poorly differentiated areas with low staining. There were no differences between chronic hepatitis B or C patients ($p>0.05$, all proteins). These data can be seen in figure 2 and table 3.

DISCUSSION

In present study, we evaluated the expression of TLR2 and TLR4 in liver samples from patients in each stage of virus-induced hepatic IFC sequence. We found an increased TLR2 and TLR4 mRNA and proteins expression in virus-induced chronic hepatitis and cirrhosis and a maintained TLR2 and TLR4 proteins expression in virus-induced hepatocarcinoma.

Although there are several animal and in vitro studies implicating TLRs in the pathogenesis of hepatic IFC sequence, studies using diseased human liver tissue are scarce. Hepatic expression of TLR2 was shown to be maintained in early and late stage biliary atresia [25] and HCV cirrhosis [26-27], increased in primary biliary cirrhosis (PBC) and non-alchoolic steatohepatitis (NASH) [28] and decreased [27] or maintained [26] in alcoholic cirrhosis. Hepatic expression of TLR4 was shown to be maintained in early and late stage biliary atresia [25], alcoholic and HCV cirrhosis [26-27], and increased in PBC [28] and NASH [28-29]. Besides contradictory results, none of these studies have evaluated simultaneously TLR expression in different stages of liver disease.

To our knowledge the present study is the first to evaluate hepatic TLR2 and TLR4 expression in different stages of virus-induced hepatic IFC sequence. We found an increased TLR2 and TLR4 mRNA and proteins expression in virus-induced chronic hepatitis and cirrhosis and a maintained TLR2 and TLR4 proteins expression in virus-induced hepatocarcinoma. Thus upregulation of TLR2 and TLR4 is an early and persistent event in virus-induced hepatic IFC sequence.

Regarding TLR2 and TLR4 proteins expression, the differences between the groups were more evident in terms of intensity of expression (which reflects the level of expression per cell) than in terms of grade of expression (which reflects the number of cells expressing the protein). The intensity of TLR2 and TLR4 proteins expression was in line with TLR2 and 4 mRNA expression, while the grade of TLRs expression changed little between the groups, not accompanying the changes in mRNA expression. This finding suggests that virus-induced hepatic IFC sequence is associated with changes in

the level of TLR2 and TLR4 proteins expression per cell and not with changes in the number of cells expressing these proteins.

Interestingly we found a smaller TLR2 and TLR4 proteins expression in hepatocarcinoma group when compared with hepatitis and cirrhosis groups. Although we have no definite explanation for the reduced expression of TLR2 and TLR4 in hepatocarcinoma cells, we believe it is likely a consequence of loss of differentiation of hepatocarcinoma cells. This is suggest by the finding that in most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining alternating with poorly differentiated areas with low staining. This finding likely means that at late stages of hepatic IFC sequence, the role of these receptors in this sequence becomes smaller.

Our study has, however, some limitations. Firstly, most patients included in control group have evidence of NAFLD and it was demonstrated that NAFLD is associated with increased hepatic TLR2 and TLR4 mRNA expression [28-29]. This suggests that the increase in hepatic expression of TLR2 and TLR4 in chronic hepatitis, cirrhosis and hepatocarcinoma may be, in fact, underestimated. Moreover, hepatitis, cirrhosis and hepatocarcinoma groups included both patients with HBV infection or HCV infection. Nonetheless, statistical analysis revealed no difference between HBV and HCV patients. Moreover, as we included only patients with virus-induced chronic hepatitis in this study, our data cannot be generalized to other chronic hepatic diseases that follow IFC sequence. Due to logistical reasons, related to sampling, we were unable to evaluate TLR2 and TLR4 mRNA expression in hepatocarcinoma tissue. Nonetheless maintained TLR2 and TLR4 mRNA expression is suggested by immunohistochemical data.

In the present study we have not explored the mechanisms underlying increased hepatic expression of TLR2 and TLR4. Nonetheless, previous studies have shown that HBV and HCV may upregulate TLR2 and TLR4 through direct and indirect mechanisms. In vitro studies have shown that HCV nonstructural protein NS5A up-regulates TLR4 expression and that HBeAg upregulates TLR2 expression [23,30]. In chronic hepatitis B and C, besides up-regulated expression of TLR2 and TLR4 by

the virus, other factors such as augmented exposure to their ligands can also contribute to increased activation of these TLRs, especially in later stages of hepatic fibrosis and cirrhosis. In fact, several studies have demonstrated that bacterial translocation is increased in patients with cirrhosis, resulting in augmented exposure of hepatic TLRs to their ligands [10-11].

Herein we did not search for liver cell-specific expression of TLR2 and TLR4, but instead we have focused on total hepatic mRNA expression and protein expression of hepatocytes. This may be an important issue as it has been demonstrated that HCV and HBV may affect TLRs expression in a cell-specific manner [2-5]. Nevertheless, the immunohistochemistry that we performed in this study suggests that, at least in part, this increase in TLRs expression occurs significantly in hepatocytes.

Previous studies on human samples have shown that hepatic expression of TLR2 and TLR4 in HCV cirrhosis was unchanged as compared with controls, which is in disagreement with our results [26-27]. The reasons for this disagreement are unclear, but our data are consistent with a previous *in vitro* study showing that hepatocyte-specific transgenic expression of the HCV nonstructural protein NS5A up-regulates TLR4 expression [23]. Regarding chronic hepatitis B, Visvanathan *et al* [30] have shown that expression of TLR2 on hepatocytes and Kupffer cells was significantly reduced in patients with HBeAg-positive chronic hepatitis B in comparison with HBeAg-negative chronic hepatitis B and controls, whereas it was significantly increased in HBeAg-negative chronic hepatitis B compared with controls. The level of TLR4 expression did not differ significantly among the groups. Down-regulation of TLR2 was also demonstrated in HepG-2 cells transduced with wild-type HBV (HBeAg-positive) but not in cells transduced with precore mutant HBV (HBeAg-negative). Regarding TLR2, our data are consistent with the study by Visvanathan *et al* since most of our chronic hepatitis B patients were HBeAg-negative and have increased TLR2. We could not compare HBeAg-positive and HBeAg-negative patients due to limited number of HBeAg-positive patients in our study.

In order to clarify TLR2- and TLR4-induced proinflammatory genes expression we also studied NF-κB, TNF-α and COX-2 mRNA expression. We found that hepatic TNF-α and COX-2 mRNA

expression is increased in virus-induced chronic hepatitis and cirrhosis, whereas hepatic NF-κB mRNA is increased in virus-induced chronic hepatitis, but maintained in virus-induced cirrhosis. This is an interesting finding since these proinflamatory genes have been implicated in hepatic inflammation, fibrogenesis and carcinogenesis interplay [1, 31-32]. Although we have not investigated functionality of TLR2 and TLR4, increased expression of TLR2 and TLR4 proteins and of NF-κB, TNF-α, COX-2 (key mediators of TLR2 and TLR4 signaling pathway) mRNA expression suggest augmented signaling of TLR2 and TLR4. When comparing hepatitis with cirrhosis we did find a tendency for a lower expression of inflammatory genes mRNA, still we cannot exclude that this tendency is not related with a higher inflammatory cell infiltrate observed in the hepatitis group. In fact, we did not find any clear tendency in the hepatocyte immunohistochemistry results, suggesting that the difference between the 2 groups, if any, is not significant.

In summary, in patients with HCV or HBV chronic infection, hepatic expression of TLR2 and TLR4 is increased in chronic hepatitis and cirrhosis and is maintained in hepatocarcinoma. This is associated with increased TLR2 and TLR4-induced proinflammatory genes expression. Overall, this study suggests that TLR2 and TLR4 may be key players in human hepatic IFC sequence associated with viral chronic hepatitis.

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CONFLICT OF INTEREST STATEMENT:

The Authors declare that there is no conflict of interest.

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Table 1 - Specific PCR primers pairs for the studied genes.

Gene	Primers
GAPDH	F: 5' – GGT GGT CTC CTC TGA CTT CAA CA – 3' R: 5' – GTT GCT GTA GCC AAA TTC GTT GT – 3'
TLR-2	F: 5' – GAT CCC AAC TAG ACA AAG ACT – 3' R: 5' – CTG CGG AAG ATA ATG AAC ACC – 3'
TLR-4	F: 5' – CCA TAA AAG CCG AAA GGT GAT TGT – 3' R: 5' – AGA TGT GCC GCC CCA GGA C – 3'
NF-κB	F: 5' – CCT GGA TGA CTC TTG GGA AA – 3' R: 5' – TCA GCC AGC TGT TTG ATG TC – 3'
COX-2	F: 5' – ACC GGG GGT ATA CTA CGG TC – 3' R: 5' – ACG GGC CCT ATT TCA AAG AT – 3'
TNF-α	F: 5' – GGT TTG CTA CAA CAT GGG CTA – 3' R: 5' – AAG AGT TCC CCA GGG ACC TCT C – 3'

F-Forward primer; R-Reverse primer

Table 2 - Baseline characteristics of patients.

Group Parameter	Control	Hepatitis	Cirrhosis	Hepatocarcinoma
n	15	22	14	10
Age	48±5	41±2	48±3	72±2
Male/Female	8/7	10/12	9/5	7/3
AST (U/L) (10-36 U/L)[1]	50±7[#]	33±4[#]	80±14	35±6[#]
ALT (U/L) (10-30 U/L) [1]	81±10	44±6[*,#]	108±26	29±5[*,#]
Bilirrubin (mg/dL) (0.2-1.0 mg/dL) [1]	0.57±0.15	0.59±0.07	1.20±0.18[§]	1.43±0.10[§]
Albumin (g/dL) (3.5-5.2 g/dL) [1]	4.4±0.1	4.5±0.1	3.9±0.2[§]	3.6±0.3[§]
INR	1.04±0.02	1.08±0.02	1.20±0.03[§]	1.22±0.02[§]
HBsAg+	-	10	7	4
HBeAg+/HBeAg-	-	1/9	0/7	0/4
HBV DNA load (IU/mL)	-	3091 (<200->20000000)	2325340 (3300->20000000)	4300340 (2900->20000000)
HCVAb+	-	12	7	6
HCV genotype 1/2/3/4	-	7/3/0/2	4/1/1/1	5/0/0/1
HCV RNA load (IU/mL)	-	834037 (9232-19907580)	706025 (174044-4803266)	804029 (182055-4803266)
METAVIR Grade A0/A1/A2/A3	-	0/13/8/1	0/0/8/6	-
METAVIR Stage F0/F1/F2/F3/F4	-	6/10/6/0/0	0/0/6/8	-

Values are presented as mean ± SEM or as median and range according to the type of distribution. [1]Normal range. [#]$p<0.05$ vs cirrhosis group; *$p<0.05$ vs control group; [§]$p<0.05$ vs control and hepatitis groups.

Table 3 - Immunohistochemichal evaluation of TLR2 and TLR4: grade and intensity of expression.

Group / Parameter	Control (n=15)	Hepatitis (n=22)	Cirrhosis (n=14)	Hepatocarcinoma (n=10)
TLR2 Grade (95%CI)	3.0	3.0	2.87 (2.80-2.94)	2.2 (2.00-2.40)
TLR2 Intensity (95%CI)	1.73 (1.55-1.91)	2.27 (2.17-2.37)	2.37 (2.25-2.49)	1.9 (1.73-2.07)
TLR4 Grade (95%CI)	3.0	3.0	2.87 (2.80-2.94)	2.6 (2.44-2.76)
TLR4 Intensity (95%CI)	1.8 (1.63-1.97)	2.41 (2.31-2.51)	2.44 (2.22-2.36)	2.1 (1.87-2.33)

Values are presented as mean (95%CI).

LEGENDS

Figure 1 - mRNA quantification of TLR2, TLR4, NF-κB, TNF-α and COX-2. Levels of mRNA are expressed as arbitrary unit (AU) set as the average value of control group, after normalization for GAPDH. Results are presented as mean ± standard error of mean (SEM). *$p<0.05$ vs control group; $^{\#}p<0.05$ vs cirrhosis group.

Figure 2 - Immunohistochemical evaluation of TLR2 (left) and TLR4 (right). A) and B) - low power field magnification for TLR2 and 4 in control group: there is a higher intensity staining in acinar zones 1 and 3 than in acinar zone 2. C) and D) - high power field magnification for acinar zone 2 in control group showing sparse citoplasmatic staining. E) and F) – high and low power field magnification, respectively, in hepatitis group (acinar zones 1 and 2) showing diffuse cytoplasmatic staining (in contrast with A-D images). G) and H) - low power field magnification in cirrhosis group: all hepatocytes are stained with a diffuse cytoplasmic staining. I) and J) - low power field magnification in hepatocarcinoma group: low intensity staining in poorly differentiated areas of hepatocarcinoma, with multiple bizarre cells (I) in contrast with nodular well differentiated areas with high intensity staining in hepatocarcinoma cells (J). In most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining (J) alternating with poorly differentiated areas with low staining (I).

Figure 1

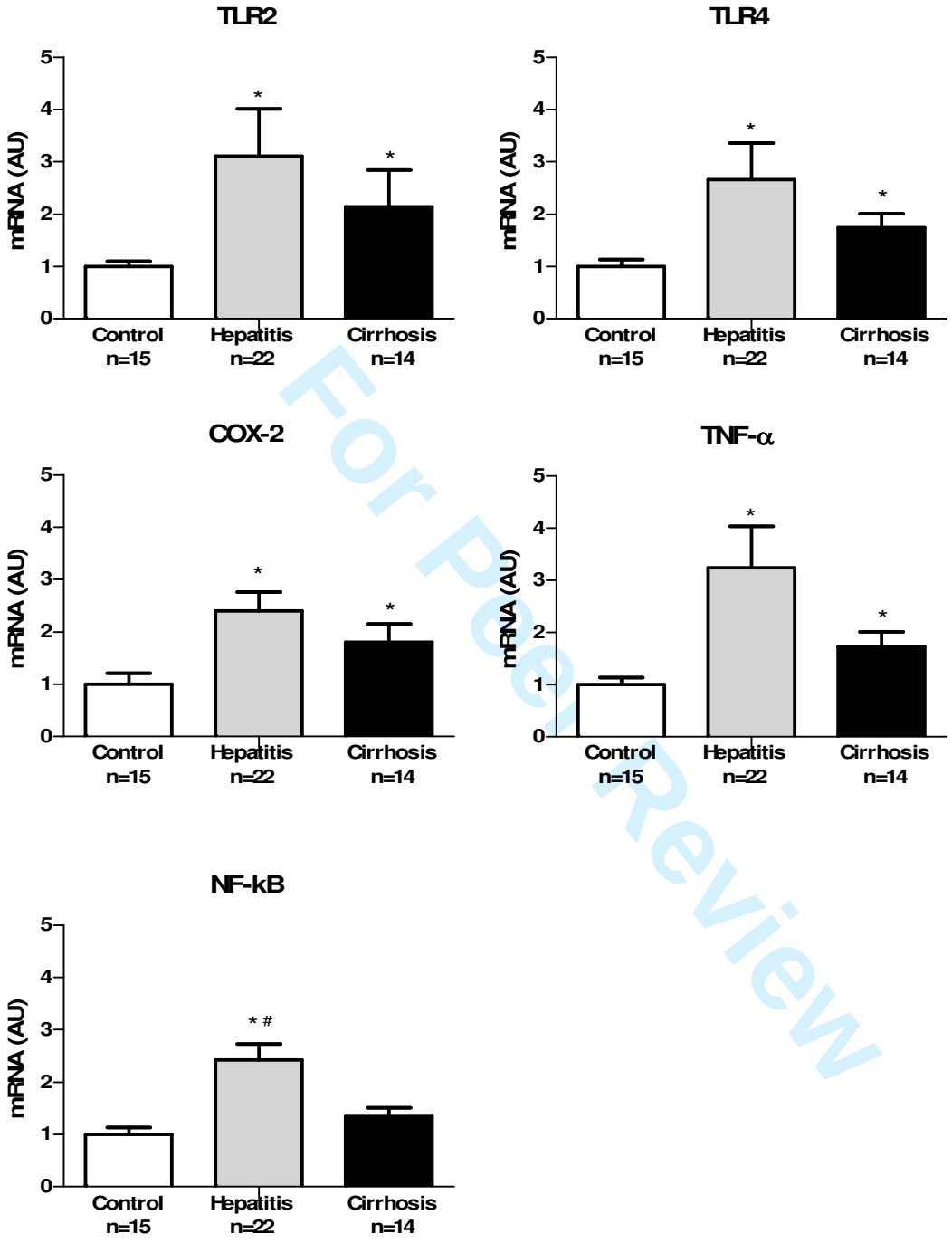
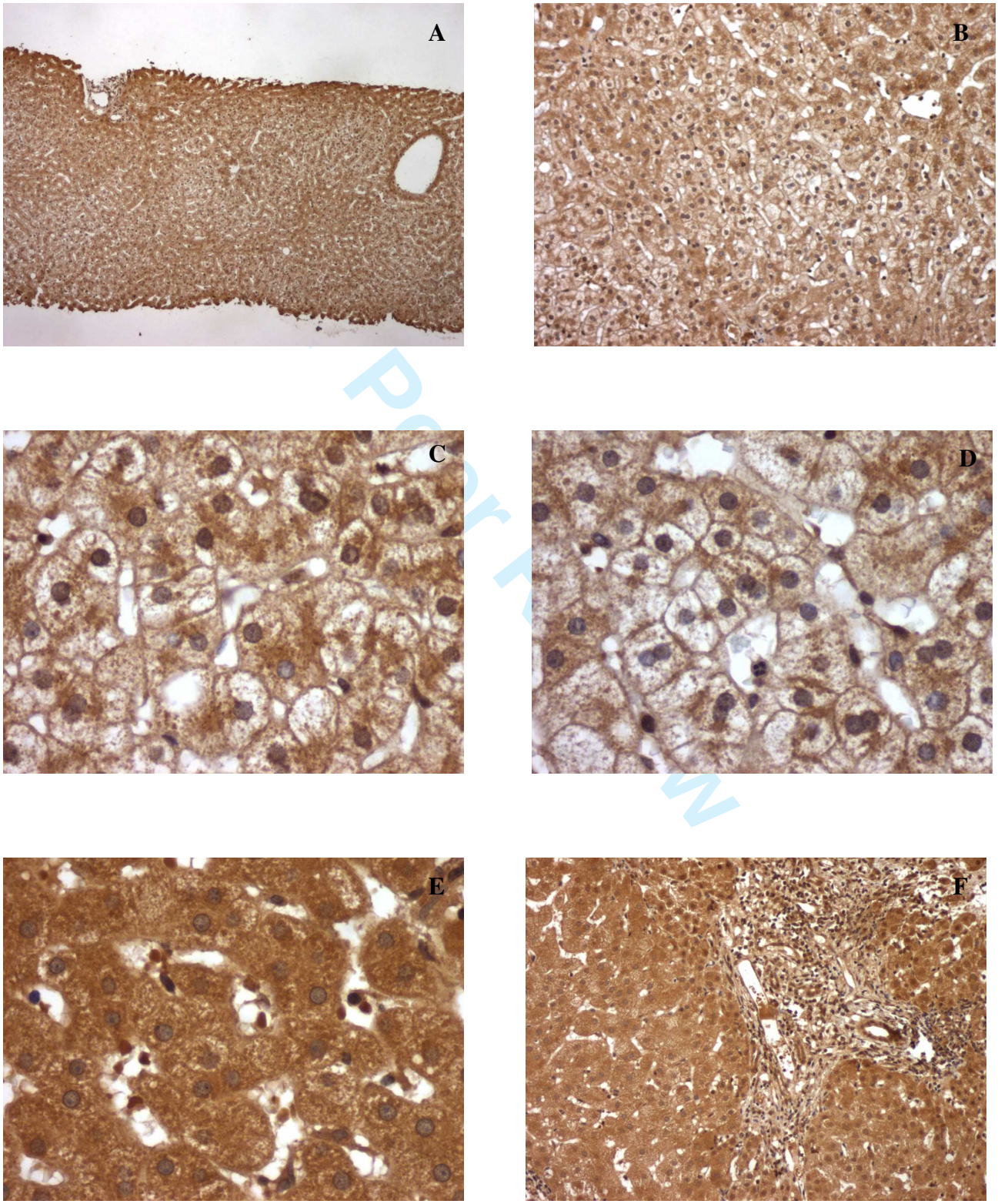
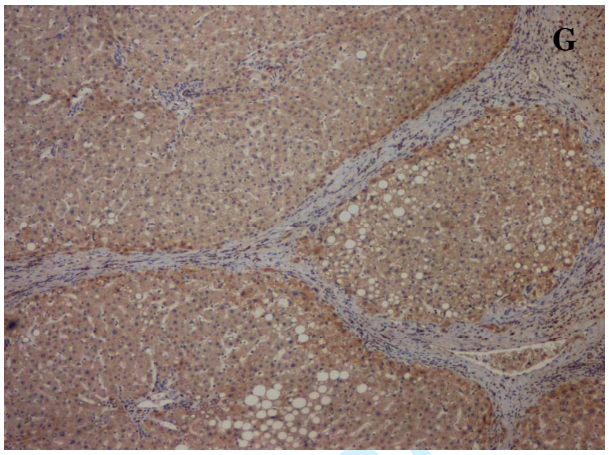
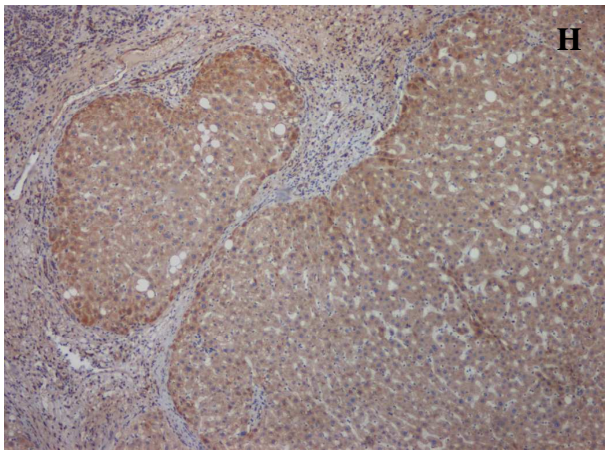


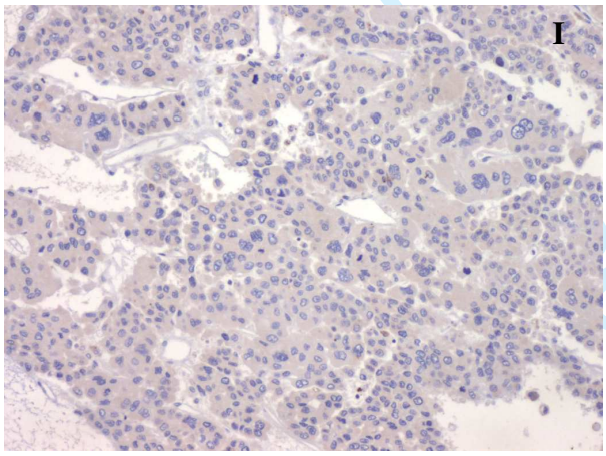
Figure 2

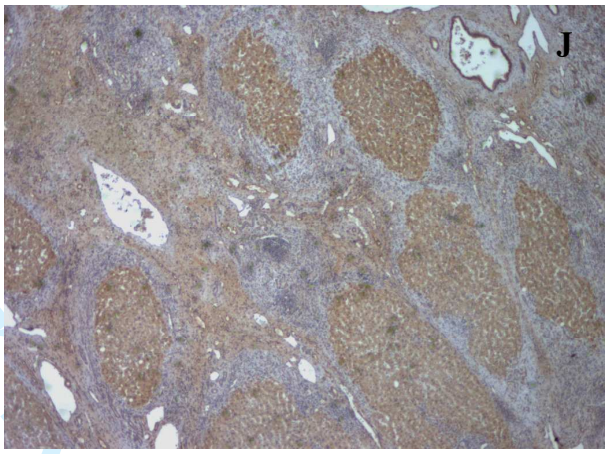
TLR2 **TLR4**

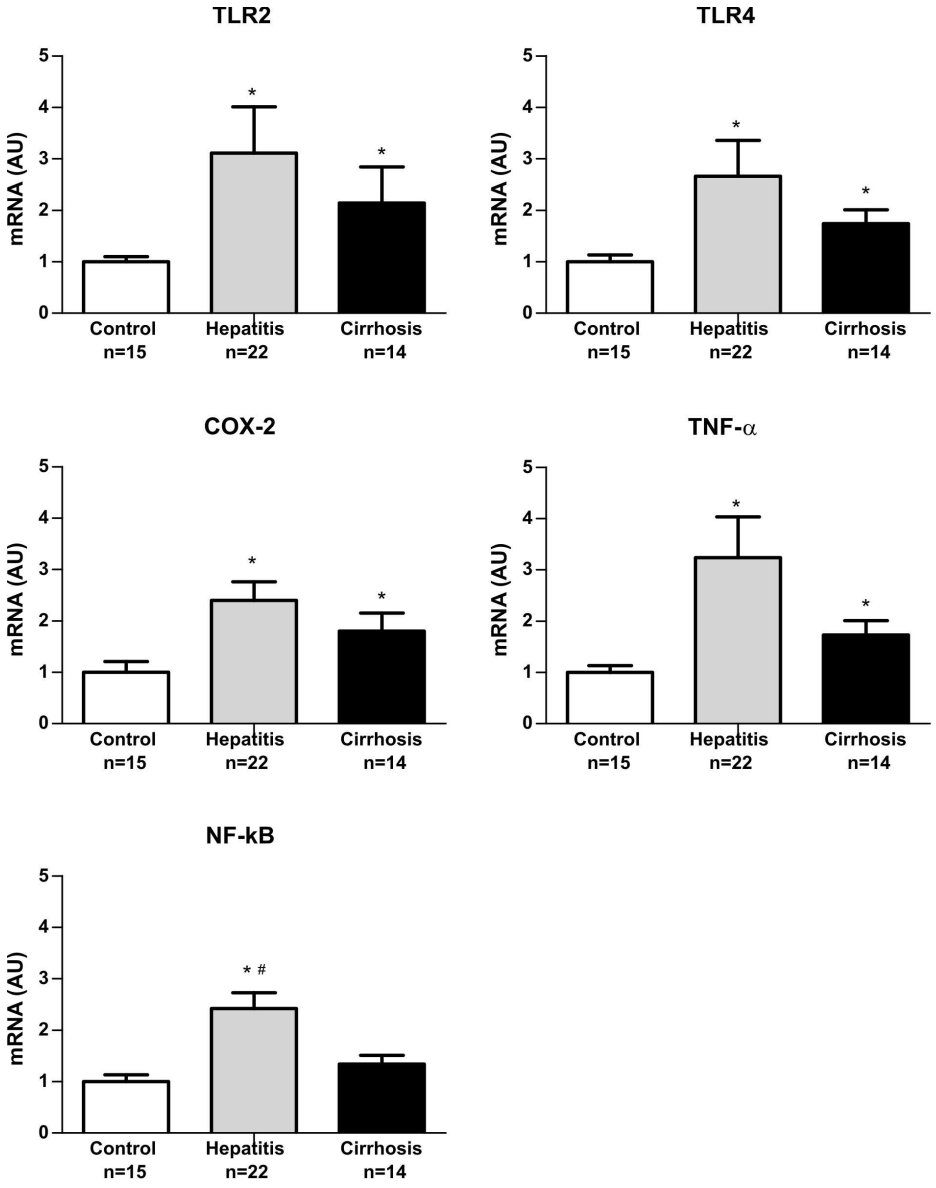












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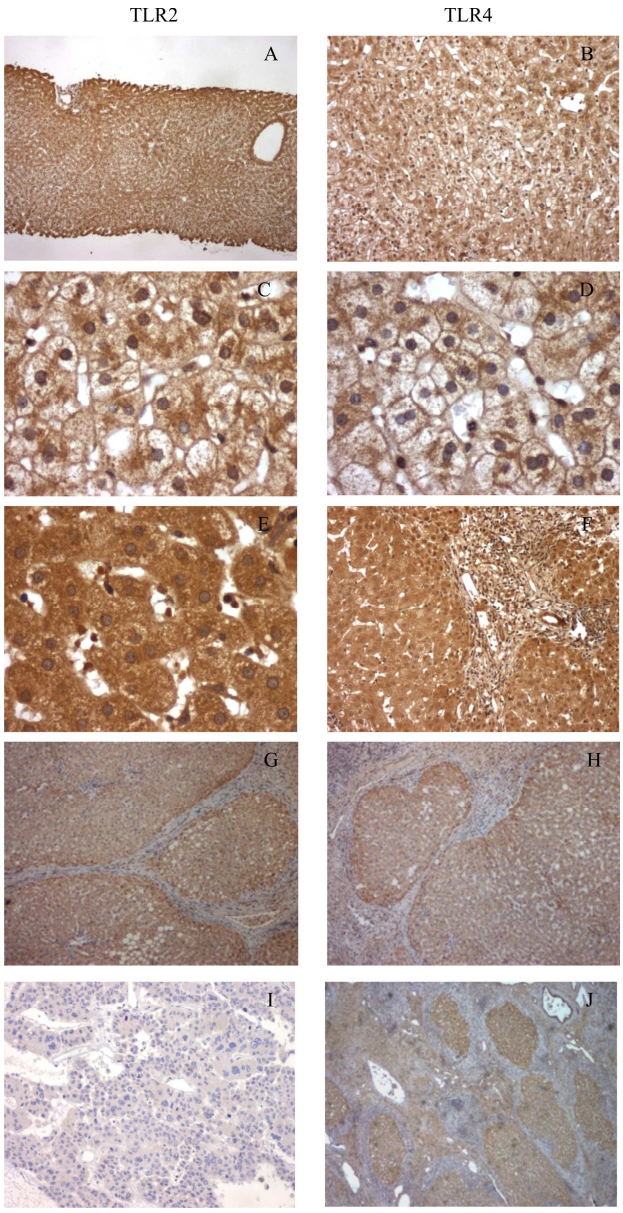

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Dear Editor,

We are sending a revised version of the manuscript ID INI-11-0005 entitled “Increased hepatic expression of toll-like receptors (TLR) 2 and 4 in the hepatic inflammation-fibrosis-carcinoma (IFC) Sequence”. The manuscript was revised throughout to comply with the reviewers' comments (changes highlighted with yellow). Point-by-point response to comments from the reviewers is provided below:

Reviewer #1: An important point is an adequate control group. Although the perfect human control does not exist and some compromises have to be accepted in studies on human samples, every effort needs to be made to minimize confounding factors. The author’s control group is inadequate, too heterogeneous and more resembles a NASH/NAFLD group where already significant inflammation is present. One can accept subjects with disturbed liver function tests as controls but the liver should be almost normal (at least no inflammation) on histology which is the case in only one single patient.

Comment #1: It is very difficult to get normal human liver samples, as we only can perform liver biopsies in patients with suspected liver disease, which is confirmed in almost all cases. So we have to accept some compromises, as we did in our study. We agree with reviewer in that our control group is not the perfect control group and this is now discussed in “Conclusion” section. In fact, most patients included in control group had evidence of NAFLD which has been associated with increased hepatic TLR2 and TLR4 mRNA expression. Nonetheless, we still were able to find an increased hepatic expression of TLR2 and TLR4 mRNA expression in chronic hepatitis and cirrhosis groups compared with control group. So, the major disadvantage of include NAFLD patients in control group would be an underestimating of the differences between control and other groups.

Reviewer #1: The sequence inflammation-fibrosis- cirrhosis is not really investigated in the study. To do so, the viral hepatitis group needs to be subdivided into 3 sub-groups: one without fibrosis, one with significant fibrosis (F2), one with advanced fibrosis/cirrhosis (F3/F4).

Comment #2: In fact, as presented in table 2, chronic hepatitis group included 6 patients without fibrosis (F0), 10 patients with minimal fibrosis and 6 patients with significant fibrosis (F2). We have decided to include all these patients in chronic hepatitis group as we didn´t find any difference between them regarding TLR mRNA and protein expression. We now include the following sentence in the Results: "We didn´t find any difference or tendency when comparing genetic profile between F0, F1 or F2 patients ($p>0.05$, all genes)."

Reviewer #1: Point 2 is important because mRNA expression of almost all investigated factors shows a clear, albeit non significant, trend towards lower levels in cirrhosis the physiopathological significance of which remains unclear. The number of cirrhotic patients also needs to be increased to allow a correct statistical analysis.

Comment #3: We agree with the reviewer when saying that in cirrhosis there was a trend for lower levels of mRNA expression when comparing with hepatitis group. Nevertheless, these values were clearly higher when comparing with the controls, and in our opinion that is the important point since it clearly shows an increased TLRs expression in cirrhosis. This tendency for a lower genetic inflammatory profile in cirrhosis when compared to hepatitis may be related to a lower inflammatory cell infiltrate in cirrhosis and not to a different hepatocyte mRNA expression. Protein results seem to confirm this opinion since there weren´t any differences or tendency between the IH profile of these 2 groups, suggesting similar TLRs expression in the hepatocytes. We now discuss this in the Conclusion: When comparing hepatitis with cirrhosis we did find a tendency for a lower expression of inflammatory genes mRNA, still we cannot exclude that this tendency is not related with a higher inflammatory cell infiltrate observed in the hepatitis group. In fact, we did not find any clear tendency in the hepatocyte immunohistochemistry results, suggesting that the difference between the 2 groups, if any, is not significant.

Reviewer #1: Moreover, protein data are in not in line with mRNA data. Differences in protein expression between the groups are minimal, non significant and do not change much compared with controls (which again are inadequate). The method used for quantification is rather crude and not very sensitive. If the authors really want to sustain that there is a difference in protein expression a more precise quantification, e. g. by western-blotting, is required.

Comment #4: First of all, concerning the method, we agree that it is not a perfect method, nevertheless, it was previously validated in other study with consistent results [1].
Secondly, we don´t agree with the reviewer when saying that the protein data is not in line with the mRNA. In fact, even though our control group can be criticized because it has a great number of NASH patients (which tend to underestimate our results and not to overestimate), TLRs expression was clearly lower when comparing with hepatitis (TLR2 IH intensity 1.73 vs 2.27; TLR4 IH intensity 1.8 vs 2.41, $p<0.05$) or cirrhosis (TLR2 IH intensity 1.73 vs 2.37; TLR4 IH intensity 1.8 vs 2.44, $p<0.05$). These results can be seen in table 3 and confirm a higher TLRs expression in hepatitis and cirrhosis, in agreement with the mRNA results.

Reviewer #1: Figure 2 should be divided into 2 separate figures: one for TLR 2 and one for TLR 4. In addition, a representative staining of all groups should be shown (including TLR2 in cirrhosis and HCC as well as TLR4 in controls and hepatitis).

Comment #5: In the revised manuscript we include updated figure 2 according to the reviewer´s suggestions.

Reviewer #1: Many data on HCC samples are lacking. In addition a clear distinction needs to be made between tumorous and non-tumorous tissue. The authors cannot claim that they have examined the sequence inflammation-fibrosis-carcinoma if all the PCR data on HCC samples are simply not available. These data need to be given for correct interpretation of the results.

Comment #6: In the hepatocarcinoma group, we obtained tumorous and non-tumorous liver tissue from surgical specimen. Nonetheless, evaluation of TLR protein expression was evaluated only in tumorous tissue. Non-tumorous tissue was obtained only for histological confirmation of cirrhosis."Material and methods" section was modified in order to clearly state this point. As stated in "Discussion" section, we could not give the PCR data on HCC samples, due to logistical reasons, related to sampling. Unfortunately, due to low number of hepatocarcinoma in our institutions, we will be unable to collect new samples in the next future. Even so, immunohistochemical data show that at least some degree of TLR mRNA expression is maintained in hepatocarcinoma tissue.

Reviewer #1: The statement " in hepatocarcinoma group TLR2 expression grade was inferior to that of TLR 4 " does not provide any information. One cannot compare the grade of expression of 2 different factors without normalizing each factor to its reference. In addition table 3 does not show a significant difference in the HCC group compared with any other group. So what do the authors want to tell the reader with this statement?

Comment #7: We agree with the reviewer. The statement "There were no differences between TLR2 and TLR4 expression grade and intensity in control, hepatitis and cirrhosis groups, but in hepatocarcinoma group TLR2 expression grade was inferior to that of TLR4" was removed in the revised manuscript.

Reviewer #1: How did the authors test the specificity of their immunostaining in livers? Testing the specificity of the antibody in non-liver tissue is not enough. Liver tissue is generally known to have a high proportion of non-specific background staining regardless of the quality

of the antibody. Ideally, this should be ruled out by using a blocking peptide or a non-immune IgG as additional controls.

Comment #8: We agree with the reviewer and, in fact, we did not use a blocking peptide or a non-immune IgG but we used as additional negative controls, samples without the primary antibodies that were completely negative. In our opinion, this is enough to confirm the specificity of our protocol. Moreover, this protocol has been validated elsewhere [1]. Nevertheless, for the issue of high proportion of non-specific background staining in the liver, we now used an antibody diluent (non-immune IgG, TA-125-UD; Thermo Scientific) as additional negative control in some samples - all samples were negative confirming the specificity of our protocol. It is now stated in the methods the following sentence:
"An antibody diluent (non-immune IgG, TA-125-UD; Thermo Scientific) was used in some samples as additional negative control, confirming the specificity of our protocol"

Reviewer #1: The paragraph on statistics is confusing. Which test has been used for which analysis?? Be more precise in your statements. In addition, the Chi-square test is inadequate for small sample sizes; the Fisher's exact test should be used instead.

Comment #9: The paragraph on statistics was completely revised in order to precise the statistical analysis.

Reviewer #1: The discussion clearly over interprets the results. A more humble discussion seems more appropriate given the numerous limitations of the study. The apparent discordance between mRNA data and protein data should also be discussed in more detail. Furthermore, some more details concerning the link between TLR2 and TLR4 and hepatitis B and C viruses might be useful for viruses in general are not the principal inducers of these TLRs.

Comment #10: We do not think our protein and mRNA data are discordant. The intensity of TLR2 and TLR4 proteins expression was in line with TLR2 and 4 mRNA expression, while the grade of TLRs expression changed little between the groups not accompanying the changes in mRNA expression. This finding suggests that the changes in mRNA expression in IFC sequence are linked to changes in TLRs expression per cell (reflected by intensity of expression) and not to changes in the number of cells expressing TLRs proteins (reflected by the grade of expression). A paragraph was added to "Discussion" section in order to discuss this point.
In fact, hepatitis B and C viruses are not the main inducers of TLR2 and TLR4. Nonetheless, previous studies have shown that HBV and HCV may upregulate TLR2 and TLR4 expression on hepatocytes through HBeAg and nonstructural protein NS5A, respectively. Moreover HBV

and HCV may also upregulate TLR2 and TLR4 through augmented exposure of these receptors to their ligands, especially in later stages of hepatic fibrosis and cirrhosis. In fact, several studies have demonstrated that bacterial translocation is increased in patients with cirrhosis, resulting in augmented exposure of hepatic TLRs to their ligands.
A paragraph was added to "Discussion" section in order to discuss this point.

Reviewer #1: The number of samples analysed in each group should be included in figure 1.

Comment #11: As suggested by reviewer, the number of samples analyzed in each group was now included in figure 1

Reviewer #2: As shown in Table 3, the grade and intensity of TLR2 and TLR4 expression in patients with liver cirrhosis were almost similar to those in patients with hepatitis. In contrast, TLR2 and TLR4 mRNA expression in patients with liver cirrhosis were somewhat smaller than those in patients with hepatitis as shown in Figure 1. Please explain the discrepancy between immunological analysis and mRNA expression.

Comment #1: Again we do not think there is any discrepancy between protein and mRNA data (see comment 4 and 10 to reviewer 1). We indeed had a trend to a smaller mRNA expression in cirrhosis group but it did not reach statistical significance, and this can be related to a lower inflammatory cell infiltrate in cirrhosis (see comment 3 to reviewer 1). So we have to assume that, like TLR2 and TLR4 protein expression, there is no difference between hepatitis and cirrhosis groups regarding TLR2 and TLR4 mRNA expression.

Reviewer #2: The authors described TLR2 and TLR4 expression in various stages of liver injury. Their observation is very interesting but their interpretation for these is not satisfactory. We would like to know the reason for the observed discrepancy between TLR2, TLR4 expression grade and expression intensity in the progression of liver injury.

Comment #2: Regarding TLR2 and TLR4 protein expression, the differences between the groups were more evident regarding intensity of expression than regarding grade of expression. This discrepancy has to do with the fact that the grade and intensity of protein expression reflect two different aspects of protein expression: the first reflects the number of cells expression of protein, while the second reflects the level of protein expression per cell.

This observed discrepancy likely suggests that virus-induced hepatic IFC sequence is associated with changes in the level of TLR2 and TLR4 protein expression per cell and not with changes in the number of cells expressing these proteins. A paragraph was added to "Discussion" section in order to discuss this point.

Reviewer #2: P11, 19-28 "Comparing with hepatitis and cirrhosis groups, TLR2 expression grade and intensity and TLR4 expression grade were reduced, while TLR4 expression intensity was maintained in hepatocarcinoma group." This finding for hepatocellular carcinoma should be discussed later. We would like to know the explanation why TLR2 and TLR4 expression was reduced in hepatoma cells. We would also like to know the meaning of this phenomenum in hepatocarsinogenesis.

Comment #3: We have no definite explanation for the reduced expression of TLR2 and TLR4 in hepatocarcinoma cells. Nonetheless, this likely has to do with loss of differentiation of hepatocarcinoma cells. This is suggested by the finding that in most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining alternating with poorly differentiated areas with low staining. This finding likely means that at late stages of hepatocarcinogenesis, the role of inflammation and of these receptors becomes smaller. A paragraph was added to "Discussion" section in order to discuss this point.

Reviewer #2: P11, 23-28 and Figure 2 legends: "In most samples of hepatocarcinoma tissue there were well-differentiated areas with high staining alternating with poorly differentiated areas with low staining." This interesting finding should be shown in a figure like as "figure 2 H".

Comment #4: In the revised manuscript we now include updated figure 2 according to the reviewer´s suggestions.

Reviewer #2: P 14, 42 -47: "Modulation of TLR2 and TLR4 pathway may be a potential therapeutic target for attenuate hepatic IFC sequence associated with chronic HBV or HCV infection, especially in patients not responding to antiviral therapy." This phrase is too speculative and not suitable for conclusion. There is no evidence to support this in the study. As the authors described that no correlation was seen between mRNA expression of any gene and viral load, necroinflammatory activity or transaminases levels, we cannot conclude TLR2 and TLR4 pathway are related to necroinflammatory activity.

Comment #5: The statement "Modulation of TLR2 and TLR4 pathway may be a potential therapeutic target for attenuate hepatic IFC sequence associated with chronic HBV or HCV infection, especially in patients not responding to antiviral therapy." was removed in the revised manuscript.

Minor points:

-errors in English expression were corrected

REFERENCES

1. Pimentel-Nunes P, Afonso L, Lopes P, Roncon-Albuquerque R, Jr., Goncalves N, Henrique R *et al.* Increased Expression of Toll-like Receptors (TLR) 2, 4 and 5 in Gastric Dysplasia. Pathol Oncol Res 2011; DOI 10.1007/s12253-011-9368-9.