

## **AIM OF THE WORK**

The aim of the present work was to study the role of mTOR and autophagy in the progression of HCV-related liver disease.

## MATERIAL

### **Subjects:**

The present study included 54 treatment-naïve patients with HCV-related liver disease referred to the Main Alexandria University Hospital. They were 27 patients with CHC (12 males and 15 females); 13 cirrhotic patients without HCC (10 males and 3 females) and 14 cirrhotic patients with HCC who underwent surgical resection of the tumor (9 males and 5 females). The age ranged between 20 to 53 years (mean  $\pm$  SD =  $39.70 \pm 9.97$  years) in patients with CHC, between 41 to 60 years (mean  $\pm$  SD =  $51.08 \pm 6.21$  years) in cirrhotic patients without HCC and between 35 to 69 years (mean  $\pm$  SD =  $55.43 \pm 9.52$  years) in patients with HCC.

The diagnosis of chronic HCV infection was based on seropositivity of circulating HCV antibodies, detectable serum HCV RNA and histopathological characteristics found in liver biopsies from patients with CHC, liver cirrhosis (when indicated) and the surrounding non-neoplastic liver tissues in patients with HCC. The presence of cirrhosis was determined by clinical, biochemical and ultrasonographic evidences and/or histopathological examination. The diagnosis of HCC was based on serum levels of alpha fetoprotein (AFP), ultrasonography, triphasic computed tomography (CT) and when needed, dynamic magnetic resonance imaging and was confirmed by histopathological examination of surgically-resected tumors.

Also, 15 age- and sex-matched healthy subjects with no evidence of liver disease were included in the study as control group in order to obtain the normal range of biochemical assays. They were 9 males and 6 females and their ages ranged between 25 - 56 years (mean  $\pm$  SD =  $39.93 \pm 9.58$  years).

### **Patient selection:**

Patients with HCV-related liver disease were excluded from the study if they had seropositivity for HBV infection; history of alcohol consumption; other known causes of chronic liver disease; concomitant schistosomiasis; hepatic decompensation; bleeding diathesis; chronic diseases such as diabetes mellitus, connective tissue diseases or other autoimmune diseases; other infections; other malignancy; and cardiac, respiratory or renal disease. Also, patients who had received previous antiviral treatment or locoregional or systemic therapy for HCC were not included in the study.

The study was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines. An informed consent was obtained from all subjects included in the study.

## METHODS

All patients with HCV-related liver disease were evaluated as regards:

### 1. Clinical evaluation focusing on:

The apparent duration and possible risk factors of HCV infection, symptoms and signs of chronic liver disease [right hypochondrial pain, jaundice, ascites, hepatic encephalopathy, previous gastrointestinal (GI) bleeding], liver and spleen sizes and the presence of palpable focal hepatic lesions.

### 2. Radiological examination:

Ultrasonographic and triphasic CT examination was performed for assessment of liver echopattern (normal, bright or coarse), liver and spleen size, presence of ascites and tumor characteristics in patients with HCC (maximum diameter; number of nodules; location and extension as % of the liver).<sup>(337,338)</sup>

### 3. Laboratory investigations:

Blood samples were collected from all patients on admission and from healthy subjects and the following tests were performed:

- a. **Complete blood picture.**<sup>(339)</sup>
- b. **Liver Test Profile:** Serum aspartate aminotransferase (AST), serum ALT, serum albumin, serum bilirubin, serum gamma glutamyl transpeptidase (GGT), and prothrombin activity (PA)/international normalized ratio (INR).<sup>(340)</sup>
- c. **Viral Testing:** Serum HCV antibodies, hepatitis B surface antigen and hepatitis B core antibody using enzyme linked immunosorbant assay (ELISA)<sup>(341)</sup> and serum HCV RNA levels using real time polymerase chain reaction (PCR).<sup>(342)</sup>
- d. **Serum creatinine.**<sup>(343)</sup>
- e. **Serum AFP levels** using standardized ELISA kit.<sup>(344)</sup>

### 4. Assessment of severity of liver disease:

The severity of liver disease in patients with HCV-related cirrhosis with and without HCC was graded according to Child-Pugh classification<sup>(345)</sup> and the Model for End Stage Liver Disease (MELD) score.<sup>(346)</sup>

### 5. Staging of HCC:

The staging of HCC was determined according to the Barcelona Clinic Liver Cancer (BCLC) staging system,<sup>(347)</sup> which includes the performance status test,<sup>(348)</sup> tumor status, Okuda stage<sup>(349)</sup> and liver function status. Patients with a single nodule greater than 8 cm were classified as stage B because they were considered as multinodular tumors.<sup>(350)</sup> The HCC stage was also determined according to the scoring system proposed by the Cancer of the Liver Italian Program (CLIP). This program includes hepatic function (Child-Pugh class), tumor morphology (number of nodules

and extension), serum AFP levels and presence of portal vein thrombosis. The tumors are divided into 7 stages (0 to 6).<sup>(351)</sup>

## **6. Measurement of serum mTOR levels:**

The mTOR protein levels in sera of patients with HCV-related liver disease and healthy subjects were measured using commercially-available human solid phase sandwich ELISA kit (MyBioSource, Inc., CA, USA) according to the manufacturer's instructions.

### ***Assay principle:***

The human mTOR ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody (Ab) specific to mTOR. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then, a biotinylated detection antibody specific for mTOR and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain mTOR, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm  $\pm$  2 nm. The OD value is proportional to the concentration of mTOR. The concentration of mTOR in the samples is calculated by comparing the OD of the samples to the standard curve.

### ***Reagents:***

1. Micro ELISA Plate (8 wells  $\times$  12 strips).
2. Reference Standard (2 vials).
3. Reference Standard & Sample Diluent (1 vial 20mL).
4. Concentrated Biotinylated Detection Ab (1 vial 120 $\mu$ L).
5. Biotinylated Detection Ab Diluent (1 vial 10mL).
6. Concentrated HRP Conjugate (1 vial 120 $\mu$ L).
7. HRP Conjugate Diluent (1 vial 10mL).
8. Concentrated Wash Buffer (25 $\times$ ) (1 vial 30mL).
9. Substrate Reagent (1 vial 10mL).
10. Stop Solution (1 vial 10mL).
11. Plate Sealer (5 pieces).

### ***Assay procedure*** .<sup>(352)</sup>

Serum samples were obtained from patients and healthy controls. All reagents were allowed to reach room temperature before use. All liquid reagents were gently mixed prior to use. A standard curve was run with each assay.

1. 100  $\mu$ L of standard, blank, or sample were added per well. The blank well was added with Reference Standard and Sample diluent. Solutions were added to the bottom of

micro ELISA plate well and mixed gently. The plates were covered by the sealer provided and incubated for 90 minutes at 37°C.

2. The liquid of each well was removed without washing.
3. Immediately, 100 µL of Biotinylated detection Ab working solution was added to each well and covered with the plate sealer, then incubated for 1 hour at 37°C.
4. Each well was aspirated and washed, and the process was repeated three times. Washing was done by filling each well with Wash Buffer (approximately 350 µL) using a squirt bottle, multi-channel pipette and manifold dispenser or automated washer. Complete removal of liquid at each step was assured. After the last wash, the remained wash buffer was removed by aspirating or decanting. The plate was inverted and patted against thick clean absorbent paper.
5. 100 µL of HRP Conjugate working solution was added to each well and covered with the plate sealer then incubated for 30 minutes at 37°C.
6. The wash process was repeated for five times as conducted in step 4.
7. 90 µL of Substrate Solution was added to each well, covered with a new plate sealer and incubated for about 15 minutes at 37°C (Protection of the plate from light was assured). The reaction time was controlled according to the actual color change, but not exceeding 30 minutes. When apparent gradient appeared in standard wells, the reaction was terminated.
8. 50 µL of Stop Solution was added to each well. Then, the color turned to yellow immediately. The order to add stop solution was the same as the substrate solution.
9. Determination of the OD of each well was done using a micro-plate reader set to 450 nm. The micro-plate reader was open in advance; the instrument was preheated, and the testing parameters were set.
10. A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and a best fit curve was drawn using graph paper or statistical software to generate a four parameter logistic (4-PL) curve-fit or logit-log linear regression curve. The data were linearized by plotting the log of the concentrations versus the log of the OD and the best fit line was determined by regression analysis.
11. The concentration of samples corresponding to the mean absorbance from the standard curve was calculated.

## **VI. Histopathological examination:**

Liver biopsy was performed in 30 patients with chronic HCV infection (all patients with CHC and three cirrhotic patients without HCC). Representative samples of surgically-resected HCCs and surrounding non-neoplastic liver tissues were also obtained. Tissue specimens were fixed in 10% solution of formalin, embedded in paraffin and subsequently stained with hematoxylin and eosin stain for routine histopathological examination. The histological activity grade (A0-A3) and fibrosis stage (F0-F4) according to the METAVIR scoring system<sup>(48)</sup> and steatosis (graded as 0 = absent; 1 = mild, less than one third; 2 = moderate, one third to two thirds and 3 = marked, more than two thirds),<sup>(353)</sup> were evaluated in liver specimens from patients with chronic HCV infection and the surrounding non-neoplastic liver tissues. The diagnosis

of HCC was confirmed by histopathological examination and the tumor was graded according to Edmonson and Steiner's criteria.<sup>(354)</sup>

**Immunohistochemical staining:**

Immunohistochemical staining of liver specimens was performed using Ultravision Avidin-Biotin Detection System (Thermo Fisher Scientific, Fremont, CA, USA). The tissue sections (5µm-thick) were placed on positively-charged or amino-propyl-tri-ethoxy-silane coated slides glass slides, dried to remove any water and de-waxed in xylene and ethanol over 15-20 minutes. This was followed by rehydration of the slides through a series of graded alcohol to water. Endogenous peroxidase activity was blocked by incubating the sections in two changes of 3% (v/v) hydrogen peroxide in phosphate buffered saline (PBS) (pH 7.4) for 10 minutes at room temperature. The next step was antigen retrieval. Heat mediated antigen retrieval was the method used where the slides were heated in 10 mM sodium citrate buffer, pH 6.0 at 95 -100°C for 10 minutes then removed from heat and let stand at room temperature in buffer for 20 minutes, then, rinsed in Tris-buffered saline for 1 minute. Tissue sections were incubated with primary antibodies for 30 minutes at room temperature. The following primary antibodies were used:

- ***Anti-human mTOR antibody*** (Rabbit anti-human mTOR polyclonal antibody, MBS301883, MyBiosource, Inc., CA, USA) at a dilution of 1:30.
- ***Anti-human Atg5 antibody*** (Rabbit anti-human Atg5 polyclonal antibody, MBS150643, MyBiosource, Inc., CA, USA) at a dilution of 1:500 as a marker for autophagy.

The slides were rinsed with PBS for 5 minutes, and nonspecific antibody binding was blocked by incubation of the tissue slides with horse serum before proceeding to the primary antibodies. Secondary antibody (Biotinylated goat antipolyvalent) was applied for 1 hour, Streptavidin peroxidase was applied for 20 minutes at room temperature. Immunostaining was visualized using DAB (3, 3' Diamono-benzidine-tetrahydrochloride) chromogen (Dako, Denmark). Positive staining to the proteins was recognized under light microscope as a diffuse cytoplasmic brown color stain. Sections were counterstained with Mayer's haematoxylin solution. Replacement of the primary antibody with rabbit immunoglobulin G was used as negative control in each batch of experiment and was run in parallel.

***Assessment of mTOR and Atg5 immunostaining:***

Immunostaining results for mTOR and Atg5 were evaluated semi-quantitatively according to the percentage of positively-stained cells in non-overlapping microscopic fields. Both cytoplasmic and nuclear staining was assessed for mTOR expression, while cytoplasmic staining was assessed for Atg5 expression. The percentage of positively stained cells was determined by counting a minimum of 100 cells, and the average counts were recorded, and scored as follows: 0 = absent staining; 1 = weak, < 10% of cells were positive; 2 = moderate, 10-50% of cells were positive or 3 = strong, > 50% of cells were positive.<sup>(355,356)</sup>

## STATISTICAL ANALYSIS

Data were collected and entered into the personal computer. Statistical analysis was done using the Statistical Package for Social Sciences (SPSS version 20.0) software (IBM-SPSS Inc., New York, United States) for Windows. The data were expressed as mean  $\pm$  SD or proportions.

- Comparison between two means was performed using the non-parametric Mann-Whitney *U*-test for abnormally distributed quantitative variables.
- The one-way ANOVA test was used for comparing the three groups with *post hoc* comparisons.
- Comparison between proportions was determined by the Chi square ( $\chi^2$ ) test or Fisher's Exact test (*FET*).
- Correlations between variables were analyzed by using Pearson's correlation coefficient or Spearman's rank test as appropriate.
- Statistical significance was assessed at  $P < 0.05$ . All calculated  $P$  values were two-tailed.
- The sensitivity and specificity of serum mTOR in discriminating cirrhotic patients with and without HCC were assessed by plotting a receiver-operating characteristic (ROC) curve and determining its cut-off value.

## RESULTS

### I. Clinical and radiological evaluation:

The clinical and ultrasonographic characteristics of patients with HCV-related liver disease are shown in tables II-VII. In patients with CHC, the apparent duration of HCV infection ranged between 3-11 months (mean  $\pm$  SD = 6.00  $\pm$  2.27 months). The possible risk factors for HCV infection were previous blood transfusion in 2 (7.4%) patients, dental treatment in 2 (7.4%) patients, previous surgery in 5 (18.5%) patients, familial contact in 3 (11.1%) patients, tattooing in 3 (11.1%) patients and undefined in 12 (44.4%) patients. The presenting symptoms were right hypochondrial pain in 12 (44.4%) patients while no patient presented with jaundice. Mild to moderate hepatomegaly with bright echopattern was found in 16 (59.3 %) patients. The ultrasonographic spleen size ranged between 11-13.5 cm (mean  $\pm$  SD; 12.40  $\pm$  0.73 cm) (Table II and VI).

In cirrhotic patients without HCC, the apparent duration of HCV infection ranged between 7-15 years (mean  $\pm$  SD = 11.08  $\pm$  2.63 years). The possible risk factors for HCV infection were previous blood transfusion in 4 (30.8%) patients, previous surgery in 4 (30.8%) patients, tattooing in one (7.7%) patient and undefined in 4 (30.8%) patients. Right hypochondrial pain was found in 7 (53.8%) patients while no patient presented with jaundice, ascites, hepatic encephalopathy or previous GI bleeding. The liver was enlarged in 5 (38.5%) patients, normal in 3 (23.1%) patients and shrunken in 5 (38.5%) patients. The liver echopattern was bright in 2 (15.4 %) patients, normal in 1 (7.7%) patients and coarse in 10 (76.9%) patients. The ultrasonographic spleen size ranged between 14-17 cm (mean  $\pm$  SD; 15.19  $\pm$  0.99 cm) (Table III and VI).

In patients with HCV-related HCC, the apparent duration of HCV infection ranged between 3-14 years (mean  $\pm$  SD = 9.58  $\pm$  3.32 years). The possible risk factors for HCV infection were previous blood transfusion in 2 (14.3%) patients, dental treatment in one (7.1%) patient, previous surgery in 3 (21.4 %) patients, familial contact in one (7.1%) patient, tattooing in 2 (14.3%) patients and undefined in 5 (35.7%) patients. The presenting symptoms were right hypochondrial pain in 10 (71.4%) while no patient presented with jaundice, ascites, hepatic encephalopathy or previous GI bleeding. The liver was enlarged in 10 (71.4%) patients and coarse liver echopattern was found in all patients. The ultrasonographic spleen size ranged between 13.5-16.5 cm (mean  $\pm$  SD; 14.75  $\pm$  0.94 cm). Focal hepatic lesions were clinically palpable in 7 (50%) patients. (Table IV and VI). The maximum diameter of HCC ranged between 2.7 and 11.6 cm with a mean value of 6.19  $\pm$  2.93 cm. The tumors were uninodular in all patients and were located in the right lobe in 5 (35.7 %) patients and in the left lobe in 9 (64.3%) patients. The extension of all surgically-resected HCCs was < 50% of the liver (Table V and VII).

### II. Laboratory evaluation:

The laboratory data of patients with HCV-related liver disease are shown in tables VIII-X and of healthy subjects in table XI. Statistical comparisons between the groups are shown in table XII.

## 1. Complete blood picture:

The hemoglobin (Hb) concentration and platelet count ranged between 11.9-16.1 g/dl and 161-307  $\times 10^3$ /cmm respectively in patients with CHC (Table VIII), between 9.2-16.0 g/dl and 33-242  $\times 10^3$ /cmm respectively in cirrhotic patients without HCC (Table IX) and between 10-14.8 g/dl and 66-330  $\times 10^3$ /cmm respectively in patients with HCC (Table X), while in healthy subjects, they ranged between 12.9-16.2 g/dl and 208-411  $\times 10^3$ /cmm respectively (Table XI). The mean Hb concentration was significantly lower in cirrhotic patients without HCC and in patients with HCC than in healthy subjects ( $12.84 \pm 1.88$  g/dl and  $13.3 \pm 1.45$  g/dl vs  $14.59 \pm 1.00$  g/dl respectively) and in cirrhotic patients without HCC than in patients with CHC ( $12.84 \pm 1.88$  g/dl vs  $13.9 \pm 1.24$  g/dl) ( $P = 0.008$ ) while there was no statistically significant difference between patients with CHC and healthy subjects ( $13.9 \pm 1.24$  g/dl vs  $14.59 \pm 1.00$  g/dl) (Table XII). The mean platelet count was significantly lower in patients with CHC, cirrhotic patients without HCC and patients with HCC than in healthy subjects ( $216.44 \pm 39.06$   $\times 10^3$ /cmm,  $111.92 \pm 54.39$   $\times 10^3$ /cmm and  $162.86 \pm 70.57$   $\times 10^3$ /cmm vs  $300.20 \pm 65.18$   $\times 10^3$ /cmm respectively), in cirrhotic patients without HCC and patients with HCC than in patients with CHC and in cirrhotic patients without HCC than in patients with HCC ( $P < 0.001$ ) (Table XII).

## 2. Liver test profile:

### a. Serum liver enzymes:

The serum levels of AST, ALT and GGT ranged between 8-80 U/L, 9-140 U/L and 12.2-49.7 U/L respectively in patients with CHC (Table VIII), 23-95 U/L, 24-163 U/L and 18.6-45.2 U/L respectively in cirrhotic patients without HCC (Table IX) and 15-92 U/L, 25-198 U/L and 18.9-29.3 U/L respectively in patients with HCC (Table X), while in healthy subjects, they ranged between 17-28 U/L, 12-28 U/L and 13-29.4 U/L respectively (Table XI).

The mean serum AST levels were significantly higher in patients with CHC, cirrhotic patients without HCC and patients with HCC than in healthy subjects ( $37.81 \pm 14.77$  U/L,  $60.46 \pm 22.87$  U/L,  $54.86 \pm 22.97$  U/L vs  $21.00 \pm 3.72$  U/L respectively) and in cirrhotic patients without HCC and patients with HCC than patients with CHC. The mean serum ALT levels were significantly higher in patients with CHC, cirrhotic patients without HCC and patients with HCC than in healthy subjects ( $52.44 \pm 29.73$  U/L,  $68.85 \pm 35.84$  U/L,  $66.29 \pm 40.53$  U/L vs  $20.33 \pm 4.64$  U/L respectively) ( $P < 0.001$ ). The mean serum GGT levels were significantly higher in patients with CHC, cirrhotic patients without HCC and patients with HCC than in healthy subjects ( $25.82 \pm 8.05$  U/L,  $32.16 \pm 9.08$  U/L,  $25.41 \pm 3.23$  U/L vs  $19.39 \pm 4.76$  U/L respectively), in cirrhotic patients without HCC than in patients with CHC and patients with HCC ( $P < 0.001$ ) (Table XII).

### b. Serum albumin:

The serum albumin concentration ranged between 3.6-5.3 g/dl in patients with CHC (Table VIII), between 3.0-4.8 g/dl in cirrhotic patients without HCC (Table IX) and between 2.9-4.6 g/dl in patients with HCC (Table X), while in healthy subjects, they ranged between 3.9-5.2 g/dl (Table XI). The mean serum albumin concentration showed

a significant decrease in cirrhotic patients without HCC and patients with HCC than in patients with CHC and healthy subjects ( $3.31 \pm 0.51$  g/dl and  $3.49 \pm 0.43$  g/dl vs  $4.26 \pm 0.50$  g/dl and  $4.49 \pm 0.48$  g/dl respectively,  $P < 0.001$ ) (Table XII).

**c. Serum bilirubin:**

The serum bilirubin levels ranged between 0.60-1.37 mg/dl in patients with CHC (Table VIII), between 0.50-1.35 mg/dl in cirrhotic patients without HCC (Table IX) and between 0.58-1.7 mg/dl in patients with HCC (Table X), while in healthy subjects, they ranged between 0.30-0.90 mg/dl (Table XI). The mean serum bilirubin levels showed a significant increase in cirrhotic patients without HCC and patients with HCC than in healthy subjects ( $1.08 \pm 0.26$  mg/dl and  $0.99 \pm 0.31$  mg/dl vs  $0.71 \pm 0.16$  mg/dl respectively) and in cirrhotic patients without HCC than in patients with CHC and patients with HCC ( $1.08 \pm 0.26$  mg/dl vs  $0.85 \pm 0.21$  mg/dl and  $0.89 \pm 0.16$  mg/dl respectively) ( $P < 0.001$ ) (Table XII).

**d. Prothrombin activity:**

The PA ranged between 74.9-100.0 % of normal in patients with CHC (Table VIII), between 68-102 % of normal in cirrhotic patients without HCC (Table IX) and between 65-99 % of normal in patients with HCC (Table X), while in normal subjects values ranged between 86-100 % of normal (Table XI). The mean PA showed a significant decrease in cirrhotic patients without HCC and patients with HCC compared with healthy subjects ( $77.79 \pm 9.68$  % of normal and  $84.79 \pm 11.20$  % of normal vs  $91.87 \pm 5.26$  % of normal respectively) and in cirrhotic patients without HCC compared with patients with CHC and patients with HCC ( $77.79 \pm 9.68$  % of normal vs  $89.83 \pm 6.16$  % of normal and  $84.79 \pm 11.20$  % of normal respectively) ( $P < 0.001$ ) (Table XII).

**3. Serum alpha-fetoprotein:**

The serum AFP levels ranged between 1.2-23.5 ng/ml in patients with CHC, between 3.6-88 ng/ml in cirrhotic patients without HCC and between 11-20973 ng/ml in patients with HCC while in normal subjects, serum AFP levels ranged between 2-4.6 ng/ml (Tables VIII-XI). The mean serum AFP levels showed a significant increase in patients with HCC compared with patients with CHC, cirrhotic patients without HCC and healthy subjects ( $2436.86 \pm 5706.94$  ng/ml vs  $7.64 \pm 5.27$  ng/ml,  $33.86 \pm 26.18$  ng/ml and  $3.39 \pm 0.77$  ng/ml respectively,  $P = 0.024$ ) (Table XII).

**4. Serum HCV-RNA Level:**

The serum HCV-RNA level ranged between  $19-6500 \times 10^3$  IU/ml (mean  $\pm$  SD =  $923.27 \pm 1504.44 \times 10^3$  IU/ml) in patients with CHC, between  $52.6 - 6231 \times 10^3$  IU/ml (mean  $\pm$  SD =  $1543.53 \pm 2208.21 \times 10^3$  IU/ml) in cirrhotic patients without HCC and between  $110-2300 \times 10^3$  IU/ml (mean  $\pm$  SD =  $675.00 \pm 626.42 \times 10^3$  IU/ml) in patients with HCC (Tables VIII-X).

**III. Severity of liver disease:**

According to Child-Pugh classification, cirrhotic patients without HCC were classified as class A in 10 (76.9%) patients and class B in 3 (23.1%) patients and the

Child-Pugh score ranged between 5-7 (mean  $\pm$  SD =  $6.08 \pm 0.64$ ). Patients with HCC were classified as class A in 12 (85.7%) patients and class B in 2 (14.3%) patients and the Child-Pugh score ranged between 5-7 (mean  $\pm$  SD;  $5.71 \pm 0.73$ ). The MELD score ranged between 6-9 (mean  $\pm$  SD =  $7.62 \pm 0.87$ ) in cirrhotic patients without HCC and between 6-10 (mean  $\pm$  SD =  $8.14 \pm 1.46$ ) in patients with HCC. There were no statistically significant differences between cirrhotic patients without HCC and patients with HCC as regards Child-Pugh classification and MELD score ( $P = 0.166$  and  $P = 0.329$  respectively) (Tables XIII and XIV).

#### **IV. Staging of HCC:**

According to BCLC staging system, HCC was classified as stage A2 in 7 (50.0%) patients, stage A3 in 2 (14.3%) patients and stage B in 5 (35.7%) patients. The CLIP scoring system categorized patients with HCC as CLIP stage 0 in 5 (35.7%) patients, CLIP stage 1 in 7 (50.0%) patients and CLIP stage 2 in 2 (14.3%) patients (Tables XIII and XIV).

#### **V. Serum mTOR levels:**

The serum mTOR levels ranged between 0.8-4.4 ng/ml in patients with CHC, between 1.5-4.5 ng/ml in cirrhotic patients without HCC, between 3.7-9.5 ng/ml in patients with HCC and between 0.6-1.6 ng/ml in healthy subjects. The mean serum mTOR levels was significantly higher in patients with CHC, cirrhotic patients without HCC and patients with HCC than in healthy subjects ( $2.22 \pm 1.20$  ng/ml,  $3.49 \pm 0.79$  ng/ml and  $6.19 \pm 1.63$  ng/ml vs  $1.13 \pm 0.30$  ng/ml respectively), in cirrhotic patients without HCC and patients with HCC than in patients with CHC and in patients with HCC than in cirrhotic patients without HCC (One-way ANOVA,  $F = 57.538$ ,  $P < 0.001$ ) (Table XV, Figure 12).

By plotting ROC curve, the sensitivity and specificity of serum mTOR levels in discriminating cirrhotic patients with and without HCC were 92.9% and 100% respectively at a cut-off value of 4.55 ng/ml [Area under the curve (AUC) = 0.970] (Figure 13).

#### **VI. Histopathological examination:**

The histopathological findings and immunohistochemical analysis of liver biopsies in patients with chronic HCV infection and HCV-related HCC are shown in tables XVI – XXI and Figures 14-34.

According to METAVIR scoring system, patients with chronic HCV infection showed histological activity grade A1 in 10 (33.3%) patients, A2 in 13 (43.3%) patients and A3 in 7 (23.3%) patients while fibrosis stage was classified as F1 in 7 (23.3%) patients, F2 in 15 (50.0%) patients, F3 in 5 (16.7%) patients and F4 in 3 (10%) patients (Tables XVI and XVIII). Steatosis was absent in 5 (16.7%) patients, mild in 10 (33.3%) patients, moderate in 8 (26.7%) patients and marked in 7 (23.3%) patients (Tables XVI and XVIII, Figures 14-17).

According to Edmondson and Steiner's grading system, HCV-related HCCs were graded as grade II in 7 (50.0%) patients, grade III in 2 (14.3%) patients and grade

IV in 5 (35.7%) patients. Different histological patterns of HCCs were encountered including trabecular pattern in 5 (35.7%) patients, acinar pattern in 2 (14.3%) patients, solid pattern in 5 (35.7%) patients, solid pseudo papillary in one (7.1%) patient and mixed pattern in one (7.1%) patient (Tables XVII and XVIII, Figures 18 and 19). The surrounding non-neoplastic liver tissues showed cirrhosis (METAVIR F4) in all patients with HCC. The histological activity grade was A2 in 4 (28.6%) patients and A3 in 10 (71.4%) patients. Steatosis was mild in 2 (14.3%) patients, moderate in 11 (78.6%) patients and marked in one (7.1%) patient (Tables XVII and XVIII).

### ***Immunostaining of mTOR:***

Positive immunostaining for mTOR was detectable as cytoplasmic and/or nuclear staining in 18 (60.0%) of patients with chronic HCV infection; of them, 4 (13.3%) patients showed weak staining, 6 (20.0%) patients showed moderate staining and 8 (26.7%) patients showed strong staining (Tables XIX and XXI, Figures 20, 22-24). In HCV-related HCCs, positive mTOR immunostaining was detected in 12 (85.7%) patients; of them weak staining was found in one (7.1%) patient, moderate staining in one (7.1%) patient and strong staining in 10 (71.4%) patients. The surrounding non-neoplastic liver tissues also showed positive mTOR immunostaining in 12 (85.7%) patients; among them, weak staining was found in 3 (21.4%) patients, moderate staining in 6 (42.9%) patients and strong staining in 3 (21.4%) patients (Tables XX and XXI, Figures 25-29).

There was a significant difference in mTOR staining intensity among patients with HCV-related liver disease ( $FET = 13.096$ ,  $P = 0.032$ ). The mTOR staining intensity was significantly higher in HCV-related HCCs than in the chronic HCV infection tissues ( $FET = 7.201$ ;  $P = 0.049$ ) and the surrounding non-neoplastic liver tissues ( $FET = 8.149$ ;  $P = 0.035$ ) while no statistically significant difference was found between the chronic HCV infection tissues and the surrounding non-neoplastic liver tissues ( $FET = 4.321$ ;  $P = 0.235$ ) (Table XXI, Figure 20).

### ***Immunostaining of Atg5:***

Positive cytoplasmic immunostaining of Atg5 was detectable in 28 (93.3%) patients with chronic HCV infection; one (3.3%) patient showed weak staining, 10 (33.3%) patients showed moderate staining and 17 (56.7%) patients showed strong staining (Tables XIX and XXI, Figures 21,30,31). In HCV-related HCCs, positive Atg5 immunostaining was detected in all patients; weak staining in 5 (35.7%) patients, moderate staining in 5 (35.7%) patients and strong staining in 4 (28.6%) patients while the surrounding non-neoplastic liver tissues also showed positive Atg5 immunostaining in all patients; among them, moderate staining in 6 (42.9%) patients and strong staining in 8 (57.1%) patients (Tables XX and XXI, Figures 32-34).

There was a significant difference in the Atg5 staining intensity among patients with HCV-related liver disease with and without HCC ( $FET = 11.297$ ,  $P = 0.038$ ). The Atg5 staining intensity was significantly lower in HCV-related HCCs than in the chronic HCV infection tissues ( $FET = 8.561$ ;  $P = 0.019$ ) and the surrounding non-neoplastic liver tissues ( $FET = 6.271$ ;  $P = 0.046$ ) while no statistically significant difference was found between the chronic HCV infection tissues and the surrounding non-neoplastic liver tissues ( $FET = 1.342$ ;  $P = 0.840$ ) (Table XXI, Figure 21).

Simultaneous high expression (score 2 or 3) of both mTOR and Atg5 was detectable in hepatocytes of 11 (36.7%) patients with chronic HCV infection and in tumor cells of 6 (42.9%) patients with HCC.

## **VII. Statistical correlations:**

Statistical correlations between serum mTOR levels, intrahepatic expression of mTOR and Atg5 and other parameters in patients with chronic HCV infection in the absence of HCC and patients with HCV-related HCC are presented in Tables XXII and XXIII respectively and showed the following results:

### ***Statistical correlations in patients with chronic HCV infection (Table XXII):***

- No statistically significant correlations were found between serum mTOR levels, and the expression of mTOR and Atg5 on one hand and age and serum levels of GGT and serum HCV RNA levels on the other hand ( $P > 0.05$ ).
- The serum mTOR levels and intrahepatic mTOR expression showed significant positive correlations with serum AST levels ( $r = 0.547$ ,  $P < 0.001$  and  $r = 0.555$ ,  $P = 0.001$  respectively), serum ALT levels ( $r = 0.624$ ,  $P < 0.001$  and  $r = 0.501$ ,  $P = 0.005$  respectively) (Figures 35 and 36), the METAVIR histological activity grade ( $r = 0.379$ ,  $P = 0.039$  and  $r = 0.442$ ,  $P = 0.015$  respectively) and fibrosis stage ( $r = 0.547$ ,  $P = 0.002$  and  $r = 0.489$ ,  $P = 0.006$  respectively) (Figures 37 and 38) and steatosis grade ( $r = 0.610$ ,  $P < 0.001$  and  $r = 0.393$ ,  $P = 0.032$  respectively; Figures 39 and 40).
- The intrahepatic Atg5 expression showed significant inverse correlations with serum AST and ALT levels ( $r = -0.409$ ,  $P = 0.025$  and  $r = -0.421$ ,  $P = 0.021$  respectively; Figure 41) and the METAVIR histological activity grade and fibrosis stage ( $r = -0.595$ ,  $P = 0.001$  and  $r = -0.420$ ,  $P = 0.021$  respectively; Figure 42). There was no significant correlation between intrahepatic Atg5 expression and steatosis grade ( $P > 0.05$ ).
- The serum mTOR levels and intrahepatic mTOR expression were positively correlated ( $r = 0.507$ ,  $P = 0.004$ , Figure 43) and both showed inverse correlations with the intrahepatic Atg5 expression ( $r = -0.398$ ,  $P = 0.029$  and  $r = -0.611$ ,  $P < 0.001$  respectively, Figures 43 and 44).

### ***Statistical correlations in patients with HCV-related HCC (Table XXIII):***

- No statistically significant correlations were found between serum mTOR levels, and the expression of mTOR and Atg5 in HCCs on one hand and Child-Pugh and MELD scores and the expression of mTOR and Atg5 in the surrounding non-neoplastic liver tissues on the other hand ( $P > 0.05$ ).
- The serum mTOR levels and mTOR expression in HCCs showed significant positive correlations with serum AFP levels ( $r = 0.725$ ,  $P = 0.003$  and  $r = 0.728$ ,  $P = 0.003$  respectively; Figure 45), HCC maximum diameter ( $r = 0.618$ ,  $P = 0.018$  and  $r = 0.574$ ,  $P = 0.032$  respectively; Figure 46), CLIP stage ( $r = 0.537$ ,  $P = 0.032$  and  $r = 0.762$ ,  $P = 0.002$  respectively; Figure 47) and HCC histological grade ( $r = 0.736$ ,  $P = 0.003$  and  $r = 0.593$ ,  $P = 0.025$  respectively; Figure 48).

- The Atg5 expression in HCCs showed significant inverse correlations with serum AFP levels ( $r = -0.713$ ,  $P = 0.004$ ; Figure 49), HCC maximum diameter ( $r = -0.840$ ,  $P < 0.001$ ; Figure 50), CLIP stage ( $r = -0.836$ ,  $P < 0.001$ ; Figure 51) and HCC histological grade ( $r = -0.808$ ,  $P < 0.001$ ; Figure 52).
- The BCLC stage showed no significant relationships with serum mTOR levels ( $F = 17.200$ ;  $P = 0.054$ ) and mTOR expression in HCCs ( $\chi^2 = 5.600$ ;  $P = 0.469$ ) and an inverse relationship with Atg5 expression ( $\chi^2 = 17.200$ ;  $P = 0.002$ ).
- The serum mTOR levels and mTOR expression in HCCs were positively correlated ( $r = 0.571$ ,  $P = 0.033$ ) and both showed inverse correlations with the Atg5 expression in HCCs ( $r = -0.703$ ,  $P = 0.005$  and  $r = -0.818$ ,  $P < 0.001$  respectively).

**Table II: Clinical and ultrasonographic data of patients with chronic hepatitis C.**

| Patient No. | Age (years) | Sex | Apparent duration of HCV infection (months) | Possible Risk factors of HCV infection | Right hypochondrial pain | Jaundice | Liver size | Liver echopattern | Spleen size (cm) |
|-------------|-------------|-----|---|--|--------------------------|----------|------------|-------------------|------------------|
| 1           | 33          | M   | 5   | -                                      | +                        | -        | Enlarged   | Bright            | 12.0             |
| 2           | 37          | F   | 7   | Previous surgery                       | -                        | -        | Enlarged   | Bright            | 11.5             |
| 3           | 38          | F   | 11  | -                                      | +                        | -        | Enlarged   | Bright            | 11.0             |
| 4           | 47          | F   | 8   | Familial contact                       | -                        | -        | Normal     | Normal            | 13.0             |
| 5           | 34          | M   | 7   | -                                      | +                        | -        | Normal     | Normal            | 12.0             |
| 6           | 43          | M   | 5   | Previous surgery                       | -                        | -        | Enlarged   | Bright            | 12.5             |
| 7           | 33          | F   | 4   | -                                      | -                        | -        | Enlarged   | Bright            | 12.0             |
| 8           | 37          | F   | 6   | Dental treatment                       | +                        | -        | Normal     | Normal            | 11.5             |
| 9           | 53          | M   | 4   | -                                      | -                        | -        | Normal     | Normal            | 12.0             |
| 10          | 38          | F   | 5   | Previous surgery                       | -                        | -        | Enlarged   | Bright            | 12.5             |
| 11          | 23          | F   | 6   | -                                      | -                        | -        | Enlarged   | Bright            | 13.0             |
| 12          | 21          | F   | 3   | -                                      | +                        | -        | Normal     | Normal            | 13.0             |
| 13          | 46          | M   | 5   | Dental treatment                       | -                        | -        | Normal     | Normal            | 12.8             |
| 14          | 40          | M   | 10  | Familial contact                       | +                        | -        | Enlarged   | Bright            | 11.0             |
| 15          | 53          | F   | 6   | Tattooing                              | +                        | -        | Normal     | Normal            | 12.5             |
| 16          | 35          | M   | 7   | -                                      | +                        | -        | Normal     | Normal            | 13.0             |

**Table II (Continued): Clinical and ultrasonographic data of patients with chronic hepatitis C.**

| <b>Patient No.</b> | <b>Age (years)</b> | <b>Sex</b> | <b>Apparent duration of HCV infection (months)</b> | <b>Possible Risk factors of HCV infection</b> | <b>Right hypochondrial pain</b> | <b>Jaundice</b> | <b>Liver size</b> | <b>Liver echopattern</b> | <b>Spleen size (cm)</b> |
|--------------------|--------------------|------------|--|---|---------------------------------|-----------------|-------------------|--------------------------|-------------------------|
| 17                 | 49                 | F          | 3  | Tattooing                                     | -                               | -               | Normal            | Normal                   | 13.0                    |
| 18                 | 20                 | M          | 8  | -   | +                               | -               | Enlarged          | Bright                   | 13.5                    |
| 19                 | 37                 | F          | 4  | -   | -                               | -               | Normal            | Normal                   | 12.5                    |
| 20                 | 50                 | M          | 5  | Blood transfusion                             | +                               | -               | Normal            | Normal                   | 11.0                    |
| 21                 | 49                 | M          | 5  | -   | -                               | -               | Enlarged          | Bright                   | 12.0                    |
| 22                 | 26                 | F          | 3  | Previous surgery                              | +                               | -               | Enlarged          | Bright                   | 12.5                    |
| 23                 | 53                 | F          | 4  | Blood transfusion                             | -                               | -               | Enlarged          | Bright                   | 12.5                    |
| 24                 | 48                 | M          | 10   | Tattooing                                     | -                               | -               | Enlarged          | Bright                   | 13.0                    |
| 25                 | 51                 | M          | 9  | Previous surgery                              | +                               | -               | Enlarged          | Bright                   | 13.5                    |
| 26                 | 32                 | F          | 8  | -   | -                               | -               | Enlarged          | Bright                   | 13.0                    |
| 27                 | 46                 | F          | 4  | Familial contact                              | -                               | -               | Enlarged          | Bright                   | 13.0                    |

+ = Present; - = Absent; M = Male; F = Female; HCV = Hepatitis C virus.

**Table III: Clinical and ultrasonographic data of cirrhotic patients without hepatocellular carcinoma.**

| Patient No. | Age (years) | Sex | Apparent duration of HCV infection (years) | Possible Risk factors of HCV infection | RHP | Jaundice | Ascites | HE | Previous GI bleeding | Liver size | Liver echopattern | Spleen size (cm) |
|-------------|-------------|-----|--|--|-----|----------|---------|----|----------------------|------------|-------------------|------------------|
| 1           | 60          | F   | 8  | Previous surgery                       | +   | -        | -       | -  | -                    | Normal     | Bright            | 15.0             |
| 2           | 57          | M   | 7  | Tattooing                              | +   | -        | -       | -  | -                    | Enlarged   | Bright            | 16.5             |
| 3           | 44          | M   | 9  | -                                      | +   | -        | -       | -  | -                    | Normal     | Normal            | 17.0             |
| 4           | 52          | M   | 11   | Previous surgery                       | -   | -        | -       | -  | -                    | Shrunken   | Coarse            | 14.0             |
| 5           | 48          | M   | 14   | Previous surgery                       | -   | -        | -       | -  | -                    | Enlarged   | Coarse            | 15.0             |
| 6           | 41          | M   | 13   | Blood transfusion                      | +   | -        | -       | -  | -                    | Shrunken   | Coarse            | 14.5             |
| 7           | 53          | M   | 14   | -                                      | -   | -        | -       | -  | -                    | Enlarged   | Coarse            | 16.0             |
| 8           | 58          | M   | 15   | Blood transfusion                      | +   | -        | -       | -  | -                    | Shrunken   | Coarse            | 15.5             |
| 9           | 41          | M   | 13   | Previous surgery                       | -   | -        | -       | -  | -                    | Enlarged   | Coarse            | 14.0             |
| 10          | 53          | F   | 12   | Blood transfusion                      | +   | -        | -       | -  | -                    | Enlarged   | Coarse            | 14.5             |
| 11          | 52          | M   | 8  | -                                      | -   | -        | -       | -  | -                    | Shrunken   | Coarse            | 15.5             |
| 12          | 56          | F   | 10   | -                                      | -   | -        | -       | -  | -                    | Normal     | Coarse            | 16.0             |
| 13          | 49          | M   | 10   | Blood transfusion                      | +   | -        | -       | -  | -                    | Shrunken   | Coarse            | 14.0             |

+ = Present; - = Absent; M = Male; F = Female; RHP = Right hypochondrial pain; HE = Hepatic encephalopathy; GI = Gastrointestinal; HCV = Hepatitis C virus.

**Table IV: Clinical and ultrasonographic data of cirrhotic patients with hepatocellular carcinoma.**

| Patient No. | Age (years) | Sex | Apparent duration of HCV infection (years) | Possible Risk factors of HCV infection | RHP | Jaundice | Ascites | HE | Previous GI bleeding | Palpable Focal hepatic lesion | Liver size | Spleen size (cm) |
|-------------|-------------|-----|--|--|-----|----------|---------|----|----------------------|-------------------------------|------------|------------------|
| 1           | 60          | M   | 11   | Tattooing                              | +   | -        | -       | -  | -                    | +                             | Enlarged   | 14.0             |
| 2           | 49          | F   | -  | -                                      | -   | -        | -       | -  | -                    | -                             | Normal     | 16.0             |
| 3           | 64          | M   | 12   | Dental treatment                       | +   | -        | -       | -  | -                    | +                             | Enlarged   | 14.5             |
| 4           | 57          | F   | 12   | Blood transfusion                      | +   | -        | -       | -  | -                    | +                             | Enlarged   | 14.0             |
| 5           | 69          | M   | 13   | Previous surgery                       | -   | -        | -       | -  | -                    | -                             | Normal     | 15.5             |
| 6           | 35          | M   | 8  | Familial contact                       | +   | -        | -       | -  | -                    | +                             | Enlarged   | 14.0             |
| 7           | 62          | M   | 9  | -                                      | +   | -        | -       | -  | -                    | -                             | Enlarged   | 14.0             |
| 8           | 65          | M   | 14   | Tattooing                              | +   | -        | -       | -  | -                    | +                             | Enlarged   | 15.0             |
| 9           | 58          | M   | 10   | Previous surgery                       | +   | -        | -       | -  | -                    | +                             | Enlarged   | 14.5             |
| 10          | 46          | F   | 3  | -                                      | +   | -        | -       | -  | -                    | -                             | Normal     | 16.0             |
| 11          | 42          | M   | 5  | -                                      | +   | -        | -       | -  | -                    | -                             | Normal     | 16.5             |
| 12          | 53          | M   | 7  | Previous surgery                       | -   | -        | -       | -  | -                    | -                             | Enlarged   | 15.0             |
| 13          | 56          | F   | 11   | Blood transfusion                      | +   | -        | -       | -  | -                    | +                             | Enlarged   | 14.0             |
| 14          | 60          | F   | -  | -                                      | -   | -        | -       | -  | -                    | -                             | Enlarged   | 13.5             |

+ = Present; - = Absent; M = Male; F = Female; RHP = Right hypochondrial pain; HE = Hepatic encephalopathy; GI = Gastrointestinal; HCV = Hepatitis C virus.

**Table V: Radiological tumor characteristics in cirrhotic patients with hepatocellular carcinoma.**

| <b>Patient No.</b> | <b>Maximum diameter (cm)</b> | <b>Number of nodules</b> | <b>Location</b> | <b>Extension (% of the liver)</b> |
|--------------------|------------------------------|--------------------------|-----------------|-----------------------------------|
| 1                  | 9.5                          | 1                        | Left lobe       | <50                               |
| 2                  | 4.9                          | 1                        | Left lobe       | <50                               |
| 3                  | 5.3                          | 1                        | Left lobe       | <50                               |
| 4                  | 11.6                         | 1                        | Right lobe      | <50                               |
| 5                  | 3.6                          | 1                        | Left lobe       | <50                               |
| 6                  | 10.0                         | 1                        | Right lobe      | <50                               |
| 7                  | 2.7                          | 1                        | Left lobe       | <50                               |
| 8                  | 9.0                          | 1                        | Right lobe      | <50                               |
| 9                  | 5.5                          | 1                        | Right lobe      | <50                               |
| 10                 | 2.8                          | 1                        | Left lobe       | <50                               |
| 11                 | 5.0                          | 1                        | Left lobe       | <50                               |
| 12                 | 4.6                          | 1                        | Left lobe       | <50                               |
| 13                 | 8.5                          | 1                        | Left lobe       | <50                               |
| 14                 | 3.6                          | 1                        | Right lobe      | <50                               |

**Table VI: Mean  $\pm$  SD and frequencies of clinical and ultraasonographic data of patients with chronic hepatitis C (CHC) and cirrhotic patients with and without hepatocellular carcinoma (HCC).**

| <b>Parameters</b>  | <b>Patients with CHC (n = 27)</b>                                   | <b>Cirrhotic patients without HCC (n = 13)</b>                    | <b>Cirrhotic patients with HCC (n = 14)</b>                        |
|--|---|---|--|
| Age (years):<br>- Range<br>- Mean $\pm$ SD   | 20 - 53<br>39.70 $\pm$ 9.97   | 41 -60<br>51.08 $\pm$ 6.21  | 35 - 69<br>55.43 $\pm$ 9.52  |
| Sex:<br>- Male (%)<br>- Female (%)   | 12 (44.4)<br>15 (55.6)  | 10 (76.9)<br>3 (23.1)   | 9 (64.3)<br>5 (35.7)   |
| Apparent duration of HCV infection:<br>- Range<br>- Mean $\pm$ SD  | 3 - 11<br>6.00 $\pm$ 2.27 (months)                                  | 7 - 15<br>11.08 $\pm$ 2.63 (years)                                | 3 - 14<br>9.58 $\pm$ 3.32 (years)                                  |
| Possible risk factors:<br>- Previous blood transfusion (%)<br>- Dental Treatment (%)<br>- Previous surgery (%)<br>- Familial contact (%)<br>- Tattooing (%)<br>- Undefined (%) | 2 (7.4)<br>2 (7.4)<br>5 (18.5)<br>3 (11.1)<br>3 (11.1)<br>12 (44.4) | 4 (30.8)<br>0 (0.0)<br>4 (30.8)<br>0 (0.0)<br>1 (7.7)<br>4 (30.8) | 2 (14.3)<br>1 (7.1)<br>3 (21.4)<br>1 (7.1)<br>2 (14.3)<br>5 (35.7) |
| Right hypochondrial pain (%)   | 12 (44.4)   | 7 (53.8)  | 10 (71.4)  |
| Jaundice (%)   | 0 (0.0)   | 0 (0.0)   | 0 (0.0)  |
| Ascites (%)  | -   | 0 (0.0)   | 0 (0.0)  |
| Hepatic encephalopathy (%)   | -   | 0 (0.0)   | 0 (0.0)  |
| Previous GI bleeding (%)   | -   | 0 (0.0)   | 0 (0.0)  |
| Palpable focal hepatic lesion (%)  | -   | -   | 7 (50.0)   |
| Liver size:<br>- Enlarged (%)<br>- Normal (%)<br>- Shrunken (%)  | 16 (59.3)<br>11 (40.7)<br>0 (0.0)                                   | 5 (38.5)<br>3 (23.1)<br>5 (38.5)                                  | 10 (71.4)<br>4 (28.6)<br>0 (0.0)                                   |
| Liver echopattern:<br>- Bright (%)<br>- Normal (%)<br>- Coarse (%)   | 16 (59.3)<br>11 (40.7)<br>0 (0.0)                                   | 2 (15.4)<br>1 (7.7)<br>10 (76.9)                                  | 0 (0.0)<br>0 (0.0)<br>14 (100.0)                                   |
| Spleen size (cm):<br>- Range<br>- Mean $\pm$ SD  | 11 - 13.5<br>12.40 $\pm$ 0.73                                       | 14 - 17<br>15.19 $\pm$ 0.99                                       | 13.5 - 16.5<br>14.75 $\pm$ 0.94                                    |

**Table VII: Mean  $\pm$  SD and frequencies of radiological tumor characteristics of cirrhotic patients with hepatocellular carcinoma (HCC).**

| <b>Parameters</b>          | <b>Cirrhotic patients with HCC<br/>(n = 14)</b> |
|----------------------------|---|
| HCC maximum diameter (cm): |   |
| - Range                    | 2.7 – 11.6                                      |
| - Mean $\pm$ SD            | 6.19 $\pm$ 2.93                                 |
| Number of nodules:         |   |
| - Uninodular (%)           | 14 (100.0)                                      |
| - Multinodular (%)         | 0 (0.0)   |
| Location:                  |   |
| - Right lobe (%)           | 5 (35.7)  |
| - Left lobe (%)            | 9 (64.3)  |
| Extension (% of the liver) |   |
| - $\leq$ 50 (%)            | 14 (100.0)                                      |
| - $>$ 50 (%)               | 0 (0.0)   |

**Table VIII: Laboratory and virological data of patients with chronic hepatitis C.**

| <b>Patient No.</b> | <b>Hb (g/dl)</b> | <b>Platelet count (x10<sup>3</sup>/cmm)</b> | <b>Serum AST (U/L)</b> | <b>Serum ALT (U/L)</b> | <b>Serum GGT (U/L)</b> | <b>Serum Albumin (g/dl)</b> | <b>Serum Bilirubin (mg/dl)</b> | <b>PA (% of normal)</b> | <b>Serum AFP (ng/ml)</b> | <b>Serum HCV-RNA (x10<sup>3</sup> IU/ml)</b> |
|--------------------|------------------|---|------------------------|------------------------|------------------------|-----------------------------|--------------------------------|-------------------------|--------------------------|--|
| 1                  | 16.1             | 240   | 23                     | 26                     | 21.2                   | 5.2                         | 0.60                           | 96.6                    | 9.8                      | 26   |
| 2                  | 13.6             | 173   | 19                     | 24                     | 15.6                   | 4.2                         | 0.67                           | 97.4                    | 8.1                      | 140.3  |
| 3                  | 14.7             | 196   | 38                     | 55                     | 19.5                   | 4.2                         | 0.75                           | 91.0                    | 4.8                      | 395.2  |
| 4                  | 12.4             | 173   | 30                     | 34                     | 33.2                   | 3.8                         | 1.01                           | 89.0                    | 6.3                      | 180  |
| 5                  | 13.1             | 169   | 18                     | 41                     | 35.7                   | 4.6                         | 0.70                           | 81.0                    | 2.2                      | 3010.6                                       |
| 6                  | 14.2             | 281   | 36                     | 70                     | 38.2                   | 3.9                         | 0.90                           | 88.0                    | 4.3                      | 104.3  |
| 7                  | 13.5             | 241   | 25                     | 33                     | 37.7                   | 3.6                         | 0.60                           | 95.0                    | 3.1                      | 1400   |
| 8                  | 15.3             | 261   | 55                     | 40                     | 32.9                   | 5.1                         | 1.29                           | 86.0                    | 4.9                      | 135  |
| 9                  | 14.7             | 274   | 8                      | 9                      | 29.6                   | 3.6                         | 0.80                           | 88.0                    | 12.8                     | 412.7  |
| 10                 | 13.6             | 251   | 47                     | 49                     | 26.2                   | 5.3                         | 0.89                           | 84.0                    | 5.7                      | 1800   |
| 11                 | 13.0             | 231   | 41                     | 72                     | 22.3                   | 4.2                         | 0.60                           | 93.0                    | 11.0                     | 163.2  |
| 12                 | 13.0             | 307   | 34                     | 31                     | 24.5                   | 3.6                         | 0.75                           | 82.5                    | 9.3                      | 4300   |
| 13                 | 15.9             | 185   | 47                     | 32                     | 19.2                   | 4.1                         | 0.90                           | 100.0                   | 4.6                      | 6500   |
| 14                 | 11.9             | 198   | 31                     | 55                     | 23.7                   | 4.4                         | 1.19                           | 74.9                    | 7.8                      | 458.1  |
| 15                 | 13.3             | 195   | 68                     | 112                    | 49.7                   | 4.2                         | 0.90                           | 85.0                    | 4.6                      | 100  |
| 16                 | 14.2             | 178   | 43                     | 51                     | 25.9                   | 4.2                         | 0.82                           | 93.0                    | 1.2                      | 1830   |

**Table VIII (Continued): Laboratory and virological data of patients with chronic hepatitis C.**

| <b>Patient No.</b> | <b>Hb (g/dl)</b> | <b>Platelet count (x10<sup>3</sup>/cmm)</b> | <b>Serum AST (U/L)</b> | <b>Serum ALT (U/L)</b> | <b>Serum GGT (U/L)</b> | <b>Serum Albumin (g/dl)</b> | <b>Serum Bilirubin (mg/dl)</b> | <b>PA (% of normal)</b> | <b>Serum AFP (ng/ml)</b> | <b>Serum HCV RNA (x10<sup>3</sup> IU/ml)</b> |
|--------------------|------------------|---|------------------------|------------------------|------------------------|-----------------------------|--------------------------------|-------------------------|--------------------------|--|
| 17                 | 13.8             | 260   | 47                     | 85                     | 21.2                   | 3.6                         | 0.95                           | 80                      | 1.2                      | 106.8  |
| 18                 | 14.6             | 231   | 32                     | 29                     | 17.2                   | 4.8                         | 0.71                           | 96                      | 3.4                      | 19   |
| 19                 | 12.4             | 240   | 41                     | 43                     | 22.8                   | 4.6                         | 0.65                           | 93                      | 2.9                      | 764  |
| 20                 | 14.9             | 161   | 41                     | 41                     | 25.6                   | 4.3                         | 1.37                           | 90                      | 2.5                      | 42   |
| 21                 | 16.1             | 199   | 45                     | 93                     | 24.5                   | 4.0                         | 0.80                           | 94                      | 8.6                      | 962  |
| 22                 | 12.4             | 206   | 80                     | 140                    | 29.3                   | 4.1                         | 1.02                           | 90                      | 11.6                     | 1000   |
| 23                 | 13.0             | 231   | 38                     | 33                     | 24.5                   | 4.9                         | 0.85                           | 90                      | 11.3                     | 34   |
| 24                 | 15.7             | 192   | 31                     | 41                     | 21.7                   | 4.8                         | 0.80                           | 85                      | 18.6                     | 437.3  |
| 25                 | 14.7             | 192   | 36                     | 92                     | 24.5                   | 4.0                         | 0.70                           | 100                     | 10.6                     | 111.9  |
| 26                 | 12.8             | 262   | 28                     | 39                     | 12.2                   | 3.9                         | 0.60                           | 93                      | 23.5                     | 295  |
| 27                 | 12.6             | 198   | 39                     | 46                     | 18.6                   | 3.8                         | 1.12                           | 90                      | 11.6                     | 201  |
| Range              | 11.9 -16.1       | 161-307                                     | 8 - 80                 | 9 - 140                | 12.2 - 49.7            | 3.6- 5.3                    | 0.60 - 1.37                    | 74.9 - 100.0            | 1.2 - 23.5               | 19 - 6500                                    |
| Mean<br>± SD       | 13.91<br>± 1.24  | 216.44<br>± 39.06                           | 37.81<br>± 14.77       | 52.44<br>± 29.73       | 25.82<br>± 8.05        | 4.26<br>± 0.50              | 0.85<br>± 0.21                 | 89.83<br>± 6.16         | 7.64<br>± 5.27           | 923.27<br>± 1504.44                          |

Hb = Hemoglobin concentration; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; GGT = Gamma glutamyl transpeptidase; PA = Prothrombin activity; AFP = Alpha fetoprotein; HCV = Hepatitis C virus.

**Table IX: Laboratory and virological data of cirrhotic patients without hepatocellular carcinoma.**

| Patient No.  | Hb (g/dl)       | Platelet count (x10 <sup>3</sup> /cmm) | Serum AST (U/L)  | Serum ALT (U/L)  | Serum GGT (U/L) | Serum albumin (g/dl) | Serum bilirubin (mg/dl) | PA (% of normal) | INR            | Serum Cr (mg/dl) | Serum AFP (ng/ml) | Serum HCV RNA (x10 <sup>3</sup> IU/ml) |
|--------------|-----------------|--|------------------|------------------|-----------------|----------------------|-------------------------|------------------|----------------|------------------|-------------------|--|
| 1            | 15.7            | 242                                    | 84               | 65               | 44.7            | 3.20                 | 0.50                    | 89.3             | 1.06           | 0.70             | 9.6               | 110.0                                  |
| 2            | 14.7            | 156                                    | 92               | 53               | 45.2            | 4.80                 | 0.68                    | 86.0             | 1.15           | 0.80             | 6.0               | 52.6                                   |
| 3            | 12.8            | 153                                    | 23               | 25               | 36.3            | 3.30                 | 0.88                    | 102.0            | 1.00           | 0.73             | 3.6               | 320.0                                  |
| 4            | 12.6            | 103                                    | 54               | 51               | 32.4            | 3.50                 | 1.05                    | 72.0             | 1.02           | 0.95             | 31.0              | 102                                    |
| 5            | 13.0            | 115                                    | 95               | 102              | 37.4            | 3.20                 | 1.25                    | 70.0             | 1.10           | 1.10             | 29.0              | 512                                    |
| 6            | 12.8            | 59                                     | 67               | 74               | 21.7            | 3.40                 | 1.15                    | 71.0             | 1.05           | 0.90             | 88.0              | 2100                                   |
| 7            | 16.0            | 42                                     | 79               | 163              | 18.6            | 3.20                 | 1.28                    | 74.0             | 1.07           | 1.00             | 18.0              | 433                                    |
| 8            | 11.1            | 33                                     | 32               | 24               | 23.1            | 3.00                 | 1.30                    | 68.0             | 1.20           | 0.80             | 69.0              | 5023                                   |
| 9            | 13.2            | 112                                    | 68               | 75               | 25.9            | 3.32                 | 1.35                    | 78.0             | 1.00           | 1.10             | 48.0              | 120                                    |
| 10           | 12.0            | 105                                    | 40               | 56               | 37.7            | 2.70                 | 0.90                    | 81.0             | 1.01           | 0.78             | 12.0              | 325                                    |
| 11           | 11.0            | 136                                    | 42               | 48               | 39.9            | 3.45                 | 1.22                    | 70.0             | 1.06           | 0.80             | 62.0              | 235.3                                  |
| 12           | 9.2             | 101                                    | 58               | 75               | 33.5            | 3.30                 | 1.20                    | 74.0             | 1.03           | 1.00             | 35.0              | 6231                                   |
| 13           | 12.8            | 98                                     | 52               | 84               | 21.7            | 2.70                 | 1.25                    | 76.0             | 1.01           | 0.95             | 29.0              | 4502                                   |
| Range        | 9.2 - 16        | 33 - 242                               | 23 - 95          | 24 - 163         | 18.6 - 45.2     | 2.7 - 4.8            | 0.50 -1.35              | 66-102           | 1.0-1.2        | 0.7- 1.1         | 3.6-88            | 52.6 - 6231                            |
| Mean<br>± SD | 12.84<br>± 1.88 | 111.92<br>± 54.39                      | 60.46<br>± 22.87 | 68.85<br>± 35.84 | 32.16<br>± 9.08 | 3.31<br>± 0.51       | 1.08<br>± 0.26          | 76.10<br>± 10.37 | 1.06<br>± 0.06 | 0.89<br>± 0.13   | 33.86<br>± 26.18  | 1543.53<br>± 2208.21                   |

Hb = Hemoglobin concentration; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; GGT = Gamma glutamyl transpeptidase; PA = Prothrombin activity; INR = International normalization ratio; Cr = Creatinine; AFP = Alpha fetoprotein; HCV = Hepatitis C virus.

**Table X: Laboratory and virological data of cirrhotic patients with hepatocellular carcinoma.**

| Patient No.  | Hb (g/dl)       | Platelet count (x10 <sup>3</sup> /cmm) | Serum AST (U/L)  | Serum ALT (U/L)  | Serum GGT (U/L) | Serum albumin (g/dl) | Serum bilirubin (mg/dl) | PA (% of normal) | INR            | Serum Cr (mg/dl) | Serum AFP (ng/ml)    | Serum HCV RNA (x10 <sup>3</sup> IU/ml) |
|--------------|-----------------|--|------------------|------------------|-----------------|----------------------|-------------------------|------------------|----------------|------------------|----------------------|--|
| 1            | 12.5            | 96                                     | 60               | 59               | 28.2            | 3.2                  | 0.90                    | 88               | 1.00           | 0.9              | 509.0                | 110                                    |
| 2            | 14.0            | 180                                    | 15               | 25               | 26.8            | 4.6                  | 0.70                    | 90.1             | 1.00           | 0.7              | 625.0                | 510                                    |
| 3            | 11.5            | 96                                     | 66               | 70               | 29.3            | 3.3                  | 1.70                    | 88               | 1.13           | 0.9              | 20973.0              | 250                                    |
| 4            | 13.7            | 204                                    | 77               | 80               | 24.0            | 2.9                  | 1.00                    | 68               | 1.23           | 0.6              | 1756.8               | 600                                    |
| 5            | 14.4            | 158                                    | 45               | 59               | 28.2            | 3.2                  | 0.80                    | 92               | 1.07           | 0.8              | 169.0                | 300                                    |
| 6            | 11.2            | 330                                    | 79               | 198              | 27.0            | 2.9                  | 1.10                    | 65               | 1.30           | 0.5              | 540.0                | 500                                    |
| 7            | 10.0            | 80                                     | 23               | 54               | 18.9            | 3.8                  | 0.80                    | 88               | 1.07           | 0.9              | 15.5                 | 1600                                   |
| 8            | 14.8            | 134                                    | 23               | 36               | 19.8            | 3.7                  | 0.58                    | 84               | 1.15           | 0.8              | 626.0                | 340                                    |
| 9            | 14.4            | 66                                     | 67               | 40               | 23.1            | 3.3                  | 0.80                    | 97               | 1.07           | 1.0              | 423.0                | 400                                    |
| 10           | 14.1            | 123                                    | 71               | 62               | 25.1            | 3.6                  | 1.00                    | 99               | 1.07           | 0.9              | 35.0                 | 2300                                   |
| 11           | 13.5            | 224                                    | 92               | 68               | 28.2            | 3.4                  | 0.90                    | 98               | 1.10           | 0.8              | 19.0                 | 140                                    |
| 12           | 13.6            | 202                                    | 44               | 53               | 27.0            | 3.6                  | 1.60                    | 83               | 1.20           | 0.9              | 557.0                | 1300                                   |
| 13           | 14.7            | 198                                    | 51               | 62               | 23              | 3.7                  | 1.10                    | 79               | 1.16           | 0.8              | 7856.8               | 500                                    |
| 14           | 13.8            | 189                                    | 55               | 62               | 27.1            | 3.6                  | 0.89                    | 68               | 1.29           | 0.69             | 11.0                 | 600                                    |
| Range        | 10-14.8         | 66-330                                 | 15-92            | 25-198           | 18.9-29.3       | 2.9 - 4.6            | 0.58 - 1.70             | 65 - 99          | 1 - 1.3        | 0.5 - 1          | 11-20973             | 110-2300                               |
| Mean<br>± SD | 13.30<br>± 1.45 | 162.86<br>± 70.57                      | 54.86<br>± 22.97 | 66.29<br>± 40.53 | 25.41<br>± 3.23 | 3.49<br>± 0.43       | 0.99<br>± 0.31          | 84.79<br>± 11.20 | 1.13<br>± 0.10 | 0.80<br>± 0.14   | 2436.86<br>± 5706.94 | 675.00<br>± 626.42                     |

Hb = Hemoglobin concentration; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; GGT = Gamma glutamyl transpeptidase; PA = Prothrombin activity; INR = International normalization ratio; Cr = Creatinine; AFP = Alpha fetoprotein; HCV = Hepatitis C virus.

**Table XI: Laboratory data of healthy subjects.**

| Subject No.      | Hb (g/dl)           | Platelet count ( $\times 10^3/\text{cmm}$ ) | Serum AST (U/L)     | Serum ALT (U/L)     | Serum GGT (U/L)     | Serum albumin (g/dl) | Serum bilirubin (mg/dl) | PA (% of normal)    | Serum AFP (ng/ml)  |
|------------------|---------------------|---|---------------------|---------------------|---------------------|----------------------|-------------------------|---------------------|--------------------|
| 1                | 14.30               | 235   | 17                  | 18                  | 29.4                | 4.10                 | 0.6                     | 88                  | 2.00               |
| 2                | 13.20               | 311   | 20                  | 22                  | 18.0                | 3.90                 | 0.7                     | 91                  | 3.10               |
| 3                | 14.00               | 402   | 26                  | 17                  | 25.0                | 5.10                 | 0.9                     | 89                  | 2.50               |
| 4                | 12.90               | 268   | 18                  | 26                  | 23.0                | 4.80                 | 0.6                     | 92                  | 3.40               |
| 5                | 15.20               | 356   | 27                  | 21                  | 18.0                | 4.70                 | 0.8                     | 87                  | 4.00               |
| 6                | 13.70               | 331   | 22                  | 19                  | 17.0                | 4.30                 | 0.6                     | 100                 | 3.80               |
| 7                | 14.20               | 229   | 18                  | 24                  | 26.0                | 3.90                 | 0.7                     | 86                  | 2.90               |
| 8                | 13.60               | 411   | 21                  | 18                  | 22.0                | 4.00                 | 0.9                     | 87                  | 4.10               |
| 9                | 16.00               | 252   | 22                  | 16                  | 20.0                | 5.20                 | 0.3                     | 98                  | 3.00               |
| 10               | 15.60               | 218   | 28                  | 23                  | 18.0                | 4.20                 | 0.8                     | 100                 | 2.70               |
| 11               | 15.10               | 312   | 24                  | 27                  | 13.0                | 4.80                 | 0.8                     | 90                  | 4.60               |
| 12               | 14.80               | 208   | 19                  | 28                  | 17.0                | 4.30                 | 0.7                     | 86                  | 4.20               |
| 13               | 16.20               | 361   | 18                  | 15                  | 15.0                | 4.00                 | 0.6                     | 94                  | 3.80               |
| 14               | 14.60               | 325   | 18                  | 12                  | 14.0                | 5.00                 | 0.9                     | 90                  | 2.60               |
| 15               | 15.40               | 284   | 17                  | 19                  | 15.5                | 5.10                 | 0.7                     | 100                 | 4.20               |
| Range            | 12.9 -16.2          | 208 - 411                                   | 17 - 28             | 12 - 28             | 13 - 29.4           | 3.9 - 5.2            | 0.3 - 0.9               | 86 - 100            | 2 - 4.6            |
| Mean<br>$\pm$ SD | 14.59<br>$\pm$ 1.00 | 300.20<br>$\pm$ 65.18                       | 21.00<br>$\pm$ 3.72 | 20.33<br>$\pm$ 4.64 | 19.39<br>$\pm$ 4.76 | 4.49<br>$\pm$ 0.48   | 0.71<br>$\pm$ 0.16      | 91.87<br>$\pm$ 5.26 | 3.39<br>$\pm$ 0.77 |

Hb = Hemoglobin concentration; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; GGT = Gamma glutamyl transpeptidase; PA = Prothrombin activity; AFP = Alpha-fetoprotein.

**Table XII: Statistical comparisons between patients with chronic hepatitis C (CHC), cirrhotic patients with and without hepatocellular carcinoma (HCC) and healthy subjects as regards laboratory data.**

| Parameters                             | Patients with CHC (n = 27)    | Cirrhotic patients without HCC (n = 13) | Cirrhotic patients with HCC (n = 14) | Healthy Subjects (n =15) | F*     | P value |
|--|-------------------------------|---|--------------------------------------|--------------------------|--------|---------|
| Hb concentration (g/dl)                | 13.9 ± 1.24 <sup>b</sup>      | 12.84 ± 1.88 <sup>a</sup>               | 13.3 ± 1.45 <sup>a</sup>             | 14.59 ± 1.00             | 4.341  | 0.008   |
| Platelet count (x10 <sup>3</sup> /cmm) | 216.44 ± 39.06 <sup>abc</sup> | 111.92 ± 54.39 <sup>ac</sup>            | 162.86 ± 70.57 <sup>a</sup>          | 300.20 ± 65.18           | 30.325 | < 0.001 |
| Serum AST (U/L)                        | 37.81 ± 14.77 <sup>abc</sup>  | 60.46 ± 22.87 <sup>a</sup>              | 54.86 ± 22.97 <sup>a</sup>           | 21.00 ± 3.72             | 15.769 | < 0.001 |
| Serum ALT (U/L)                        | 52.44 ± 29.73 <sup>a</sup>    | 68.85 ± 35.84 <sup>a</sup>              | 66.29 ± 40.53 <sup>a</sup>           | 20.33 ± 4.64             | 7.781  | < 0.001 |
| Serum GGT (U/L)                        | 25.82 ± 8.05 <sup>ab</sup>    | 32.16 ± 9.08 <sup>ac</sup>              | 25.41 ± 3.23 <sup>a</sup>            | 19.39 ± 4.76             | 7.893  | < 0.001 |
| Serum albumin (g/dl)                   | 4.26 ± 0.50 <sup>bc</sup>     | 3.31 ± 0.51 <sup>a</sup>                | 3.49 ± 0.43 <sup>a</sup>             | 4.49 ± 0.48              | 21.563 | < 0.001 |
| Serum bilirubin (mg/dl)                | 0.85 ± 0.21 <sup>b</sup>      | 1.08 ± 0.26 <sup>a</sup>                | 0.99 ± 0.31 <sup>a</sup>             | 0.71 ± 0.16              | 6.899  | < 0.001 |
| PA (% of normal)                       | 89.83 ± 6.16 <sup>b</sup>     | 76.10 ± 10.37 <sup>ac</sup>             | 84.79 ± 11.20 <sup>a</sup>           | 91.87 ± 5.26             | 10.977 | < 0.001 |
| Serum AFP (ng/ml)                      | 7.64 ± 5.27 <sup>c</sup>      | 33.86 ± 26.18 <sup>c</sup>              | 2436.86 ± 5706.94 <sup>a</sup>       | 3.39 ± 0.77              | 3.356  | 0.024   |
| Serum HCV RNA (x10 <sup>3</sup> IU/ml) | 923.27 ± 1504.43              | 1543.53 ± 2208.21                       | 675.00 ± 626.42                      | -                        | 1.140  | 0.328   |

Hb = Hemoglobin; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; GGT = Gamma glutamyl transpeptidase; PA = Prothrombin activity; AFP = Alpha-fetoprotein; HCV = Hepatitis C virus.

\*One-Way ANOVA

<sup>a</sup> = Significant statistical difference from healthy subjects.

<sup>b</sup> = Significant statistical difference from patients with HCV-related cirrhosis.

<sup>c</sup> = Significant statistical difference from patients with HCV-related HCC.

**Table XIII: Child-Pugh classification and the Model for End Stage Liver Disease (MELD) score in cirrhotic patients with and without hepatocellular carcinoma (HCC) and the Barcelona Clinic Liver Cancer (BCLC) stage and the Cancer of the Liver Italian Program (CLIP) stage in patients with HCC.**

| Patient No.   | Cirrhotic patients without HCC |       |                 | Cirrhotic patients with HCC |       |                 |            |            |
|---------------|--------------------------------|-------|-----------------|-----------------------------|-------|-----------------|------------|------------|
|               | Child-Pugh                     |       | MELD score      | Child-Pugh                  |       | MELD score      | BCLC stage | CLIP stage |
|               | Score                          | Class |                 | Score                       | Class |                 |            |            |
| 1             | 6                              | A     | 7               | 6                           | A     | 6               | B          | 1          |
| 2             | 5                              | A     | 8               | 5                           | A     | 6               | A2         | 1          |
| 3             | 6                              | A     | 6               | 6                           | A     | 10              | A3         | 1          |
| 4             | 6                              | A     | 7               | 7                           | B     | 9               | B          | 2          |
| 5             | 6                              | A     | 9               | 6                           | A     | 9               | A2         | 0          |
| 6             | 6                              | A     | 8               | 7                           | B     | 10              | B          | 2          |
| 7             | 6                              | A     | 8               | 5                           | A     | 7               | A2         | 0          |
| 8             | 7                              | B     | 9               | 5                           | A     | 8               | B          | 1          |
| 9             | 6                              | A     | 8               | 6                           | A     | 7               | A2         | 1          |
| 10            | 7                              | B     | 7               | 5                           | A     | 7               | A2         | 0          |
| 11            | 6                              | A     | 8               | 6                           | A     | 7               | A2         | 0          |
| 12            | 5                              | A     | 7               | 5                           | A     | 9               | A3         | 1          |
| 13            | 7                              | B     | 7               | 5                           | A     | 10              | B          | 1          |
| 14            | -                              | -     | -               | 6                           | A     | 9               | A2         | 0          |
| Range         | 5 - 7                          | -     | 6 - 9           | 5 - 7                       | -     | 6 - 10          | -          | 0-2        |
| Mean $\pm$ SD | 6.08 $\pm$ 0.64                | -     | 7.62 $\pm$ 0.87 | 5.71 $\pm$ 0.73             | -     | 8.14 $\pm$ 1.46 | -          |            |

**Table XIV: Statistical comparisons between cirrhotic patients with and without hepatocellular carcinoma (HCC) as regards Child-Pugh classification and the Model for End Stage Liver Disease (MELD) score and the frequencies of the Barcelona Clinic Liver Cancer (BCLC) stage and the Cancer of the Liver Italian Program (CLIP) stage in patients with HCC.**

| Parameters             | Cirrhotic patients without HCC (n = 13) | Cirrhotic patients with HCC (n = 14) | Statistical test   | P value |
|------------------------|---|--------------------------------------|--------------------|---------|
| Child-Pugh:            |   |                                      |                    |         |
| - Class A (%)          | 10 (76.9)                               | 12 (85.7)                            | <i>FET</i> = 0.332 | 0.648   |
| - Class B (%)          | 3 (23.1)                                | 2 (14.3)                             |                    |         |
| - Score (Mean ± SD)    | 6.08 ± 0.64                             | 5.71 ± 0.73                          | <i>Z</i> = 1.385*  | 0.166   |
| MELD score (Mean ± SD) | 7.62 ± 0.87                             | 8.14 ± 1.46                          | <i>Z</i> = 0.976*  | 0.329   |
| BCLC stage:            |   |                                      |                    |         |
| - A2 (%)               | -                                       | 7 (50.0)                             |                    |         |
| - A3 (%)               | -                                       | 2 (14.3)                             | -                  | -       |
| - B (%)                | -                                       | 5 (35.7)                             |                    |         |
| CLIP stage:            |   |                                      |                    |         |
| - CLIP 0 (%)           | -                                       | 5 (35.7)                             |                    |         |
| - CLIP 1 (%)           | -                                       | 7 (50.0)                             |                    |         |
| - CLIP 2 (%)           | -                                       | 2 (14.3)                             |                    |         |

\*Mann-Whitney U test

*FET* = Fisher's exact test.

**Table XV: Serum mammalian target of rapamycin (mTOR) levels (ng/ml) in patients with chronic hepatitis C (CHC), cirrhotic patients with and without hepatocellular carcinoma (HCC) and healthy subjects.**

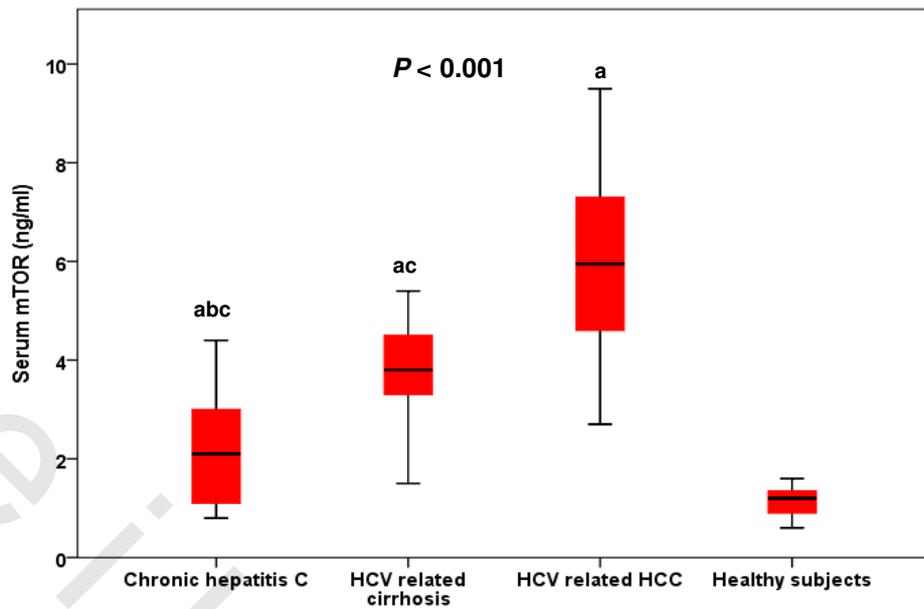
| Patient No. | Patients with CHC (n = 27) | Cirrhotic patients without HCC (n = 13) | Cirrhotic patients with HCC (n = 14) | Healthy Subjects (n =15) |
|-------------|----------------------------|---|--------------------------------------|--------------------------|
| 1           | 2.0                        | 1.5                                     | 6.5                                  | 1.2                      |
| 2           | 1.0                        | 2.8                                     | 6.4                                  | 1.4                      |
| 3           | 1.6                        | 3.1                                     | 8.3                                  | 1.4                      |
| 4           | 1.2                        | 3.7                                     | 8.0                                  | 1.2                      |
| 5           | 0.9                        | 4.5                                     | 4.6                                  | 1.6                      |
| 6           | 4.0                        | 3.4                                     | 6.2                                  | 0.9                      |
| 7           | 1.4                        | 4.3                                     | 4.8                                  | 1.2                      |
| 8           | 2.5                        | 3.3                                     | 7.3                                  | 1.5                      |
| 9           | 1.4                        | 4.1                                     | 5.5                                  | 0.6                      |
| 10          | 3.7                        | 3.5                                     | 5.5                                  | 1.1                      |
| 11          | 4.4                        | 3.8                                     | 4.6                                  | 1.3                      |
| 12          | 2.6                        | 4.2                                     | 3.7                                  | 0.8                      |
| 13          | 2.2                        | 3.2                                     | 9.5                                  | 0.7                      |
| 14          | 2.1                        | -                                       | 5.7                                  | 1.2                      |
| 15          | 4.1                        | -                                       | -                                    | 0.9                      |
| 16          | 0.9                        | -                                       | -                                    | -                        |
| 17          | 3.9                        | -                                       | -                                    | -                        |
| 18          | 0.8                        | -                                       | -                                    | -                        |
| 19          | 0.9                        | -                                       | -                                    | -                        |
| 20          | 1.0                        | -                                       | -                                    | -                        |
| 21          | 3.4                        | -                                       | -                                    | -                        |
| 22          | 4.3                        | -                                       | -                                    | -                        |
| 23          | 2.1                        | -                                       | -                                    | -                        |
| 24          | 0.9                        | -                                       | -                                    | -                        |
| 25          | 1.8                        | -                                       | -                                    | -                        |
| 26          | 2.3                        | -                                       | -                                    | -                        |
| 27          | 2.5                        | -                                       | -                                    | -                        |
| Range       | 0.8 – 4.4                  | 1.5 – 4.5                               | 3.7 – 9.5                            | 0.6 – 1.6                |
| Mean ± SD   | 2.22 ± 1.20 <sup>abc</sup> | 3.49 ± 0.79 <sup>ac</sup>               | 6.19 ± 1.63 <sup>a</sup>             | 1.13 ± 0.30              |

One-Way ANOVA;  $F = 57.538$ ;  $P < 0.001$

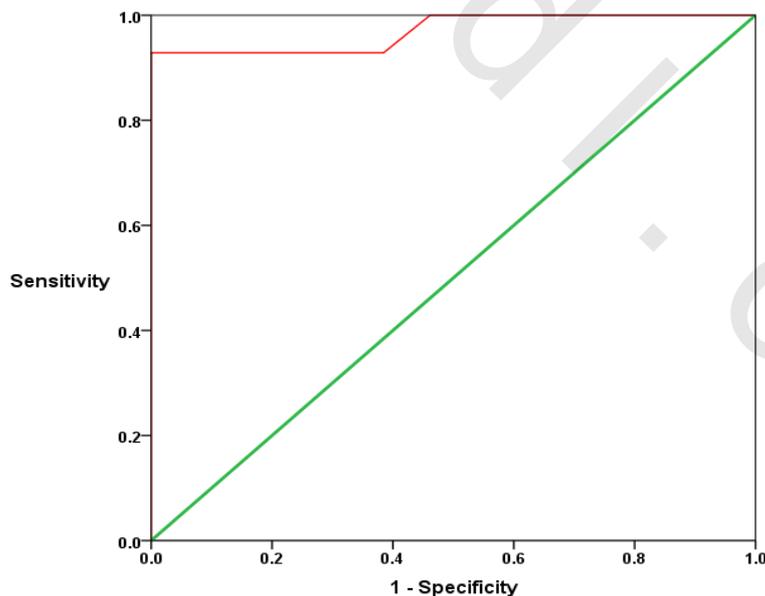
<sup>a</sup> = Significant statistical difference from healthy subjects.

<sup>b</sup> = Significant statistical difference from patients with HCV-related cirrhosis.

<sup>c</sup> = Significant statistical difference from patients with HCV-related HCC.



**Figure 12: Statistical comparisons between patients with chronic hepatitis C (CHC), cirrhotic patients with and without hepatocellular carcinoma (HCC) and healthy subjects as regards serum mammalian target of rapamycin (mTOR) levels ( $P < 0.001$ ). a = Significant statistical difference from healthy subjects; b = Significant statistical difference from cirrhotic patients without HCC; c = Significant statistical difference from cirrhotic patients with HCC.**



**Figure 13: Receiver operating characteristic curve (ROC) shows that the sensitivity and specificity of serum mammalian target of rapamycin (mTOR) levels in the diagnosis of hepatocellular carcinoma were 92.9% and 100% respectively at a cut-off value of 4.55 ng/ml [Area under the curve (AUC) = 0.970].**

**Table XVI: Histopathological findings in liver biopsies of patients with chronic hepatitis C virus (HCV) infection.**

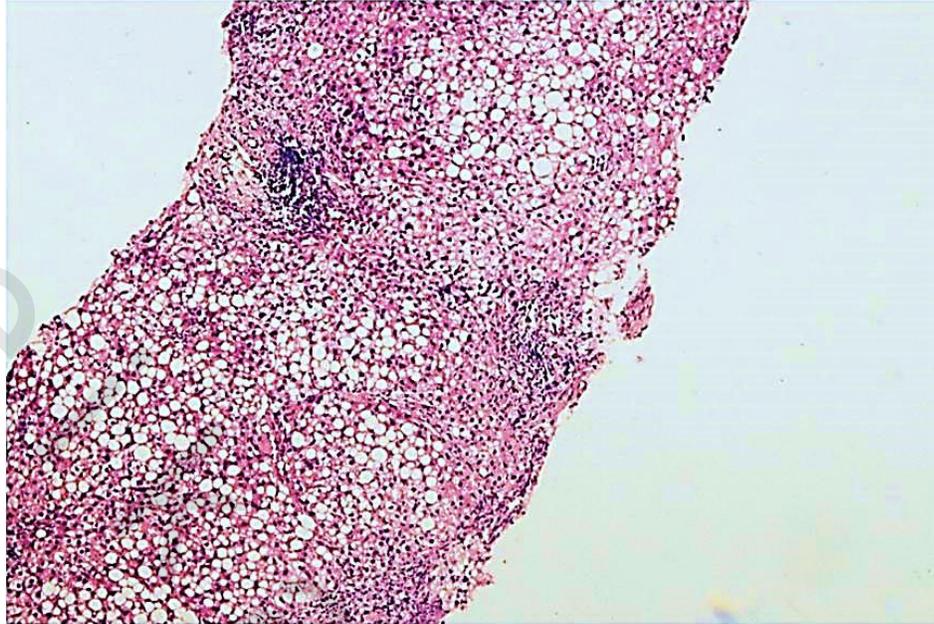
| Patient No. | METAVIR                       |                    | Steatosis grade |
|-------------|-------------------------------|--------------------|-----------------|
|             | Histologic activity grade (A) | Fibrosis Stage (F) |                 |
| 1           | A1                            | F1                 | 1               |
| 2           | A1                            | F1                 | 0               |
| 3           | A2                            | F2                 | 1               |
| 4           | A1                            | F1                 | 1               |
| 5           | A1                            | F1                 | 1               |
| 6           | A2                            | F3                 | 1               |
| 7           | A2                            | F2                 | 1               |
| 8           | A2                            | F3                 | 2               |
| 9           | A1                            | F1                 | 0               |
| 10          | A2                            | F2                 | 2               |
| 11          | A3                            | F2                 | 2               |
| 12          | A1                            | F2                 | 1               |
| 13          | A2                            | F2                 | 2               |
| 14          | A2                            | F2                 | 2               |
| 15          | A2                            | F3                 | 2               |
| 16          | A1                            | F1                 | 0               |
| 17          | A2                            | F3                 | 3               |
| 18          | A3                            | F2                 | 1               |
| 19          | A1                            | F2                 | 0               |
| 20          | A3                            | F2                 | 1               |
| 21          | A3                            | F2                 | 2               |
| 22          | A3                            | F3                 | 3               |
| 23          | A2                            | F2                 | 1               |
| 24          | A3                            | F2                 | 2               |
| 25          | A1                            | F2                 | 0               |
| 26          | A2                            | F2                 | 3               |
| 27          | A2                            | F1                 | 3               |
| 28          | A1                            | F4                 | 3               |
| 29          | A2                            | F4                 | 3               |
| 30          | A3                            | F4                 | 3               |

**Table XVII: Histopathological findings in hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) and the surrounding non-neoplastic liver tissues.**

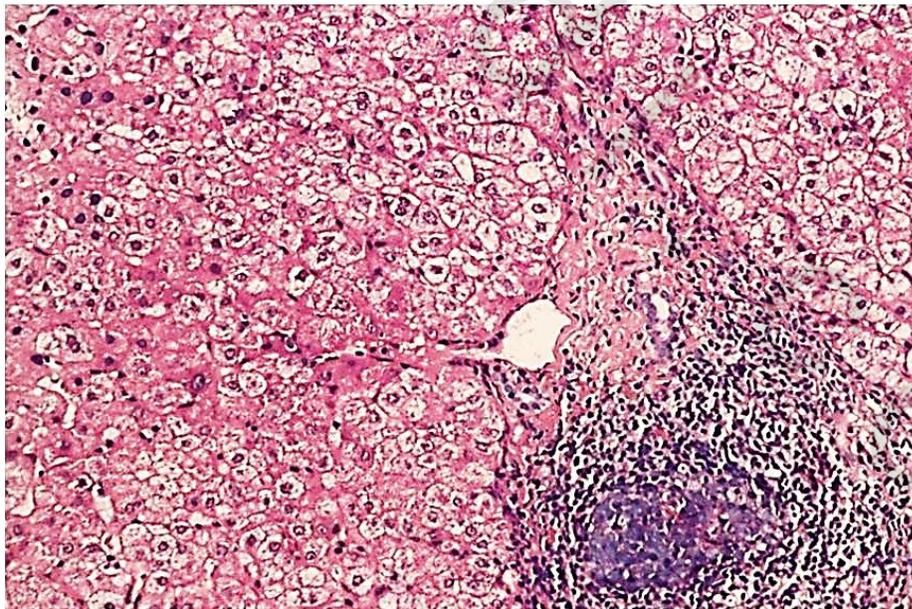
| Patient No. | HCV-related HCC    |                        | Surrounding non-neoplastic liver tissues |                    |                 |
|-------------|--------------------|------------------------|--|--------------------|-----------------|
|             | Histological grade | Pattern                | METAVIR                                  |                    | Steatosis grade |
|             |                    |                        | Histologic activity grade (A)            | Fibrosis stage (F) |                 |
| 1           | IV                 | Solid                  | A3                                       | F4                 | 2               |
| 2           | II                 | Trabecular             | A3                                       | F4                 | 2               |
| 3           | IV                 | Solid                  | A3                                       | F4                 | 2               |
| 4           | IV                 | Solid pseudo papillary | A3                                       | F4                 | 2               |
| 5           | II                 | Trabecular + Acinar    | A3                                       | F4                 | 2               |
| 6           | IV                 | Solid                  | A3                                       | F4                 | 2               |
| 7           | II                 | Acinar                 | A3                                       | F4                 | 2               |
| 8           | IV                 | Trabecular             | A3                                       | F4                 | 2               |
| 9           | III                | Solid                  | A2                                       | F4                 | 1               |
| 10          | II                 | Trabecular             | A2                                       | F4                 | 2               |
| 11          | II                 | Trabecular             | A3                                       | F4                 | 2               |
| 12          | II                 | Solid                  | A3                                       | F4                 | 2               |
| 13          | III                | Acinar                 | A2                                       | F4                 | 1               |
| 14          | II                 | Trabecular             | A2                                       | F4                 | 3               |

**Table XVIII: Frequencies of histopathological findings in liver biopsies of chronic hepatitis C virus (HCV) infection, HCV-related hepatocellular carcinoma and the surrounding non-neoplastic liver tissues.**

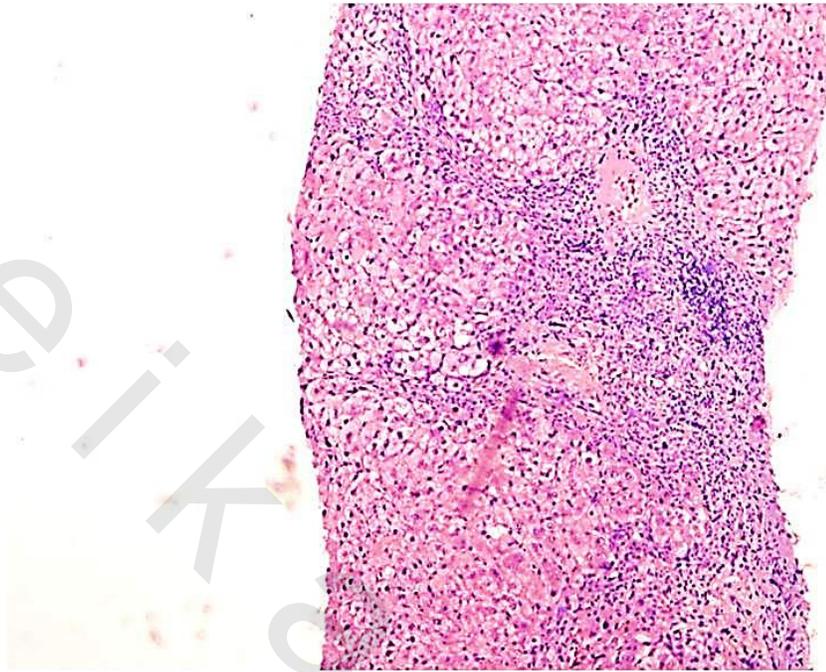
| <b>Parameters</b>                  | <b>Chronic HCV infection<br/>(n = 30)</b> | <b>HCV-related HCC<br/>(n = 14)</b> | <b>Surrounding non-neoplastic liver tissues<br/>(n = 14)</b> |
|------------------------------------|---|-------------------------------------|--|
| <b>METAVIR:</b>                    |   |                                     |  |
| <b>Histologic activity grade :</b> |   |                                     |  |
| - A1 (%)                           | 10 (33.3)                                 | -                                   | 0 (0.0)  |
| - A2 (%)                           | 13 (43.3)                                 | -                                   | 4 (28.6)   |
| - A3 (%)                           | 7 (23.3)                                  | -                                   | 10 (71.4)  |
| <b>Fibrosis stage:</b>             |   |                                     |  |
| - F1 (%)                           | 7 (23.3)                                  | -                                   | 0 (0.0)  |
| - F2 (%)                           | 15 (50.0)                                 | -                                   | 0 (0.0)  |
| - F3 (%)                           | 5 (16.7)                                  | -                                   | 0 (0.0)  |
| - F4 (%)                           | 3 (10.0)                                  | -                                   | 14 (100.0)   |
| <b>Steatosis Grade</b>             |   |                                     |  |
| - 0 (%)                            | 5 (16.7)                                  | -                                   | 0 (0.0)  |
| - 1 (%)                            | 10 (33.3)                                 | -                                   | 2 (14.3)   |
| - 2 (%)                            | 8 (26.7)                                  | -                                   | 11 (78.6)  |
| - 3 (%)                            | 7 (23.3)                                  | -                                   | 1 (7.1)  |
| <b>HCC grade</b>                   |   |                                     |  |
| - Grade II (%)                     | -   | 7 (50.0)                            | -  |
| - Grade III (%)                    | -   | 2 (14.3)                            | -  |
| - Grade IV (%)                     | -   | 5 (35.7)                            | -  |
| <b>HCC pattern</b>                 |   |                                     |  |
| - Trabecular (%)                   | -   | 5 (35.7)                            | -  |
| - Acinar (%)                       | -   | 2 (14.3)                            | -  |
| - Solid (%)                        | -   | 5 (35.7)                            | -  |
| - Solid pseudo papillary (%)       | -   | 1 (7.1)                             | -  |
| - Trabecular + Acinar (%)          | -   | 1 (7.1)                             | -  |



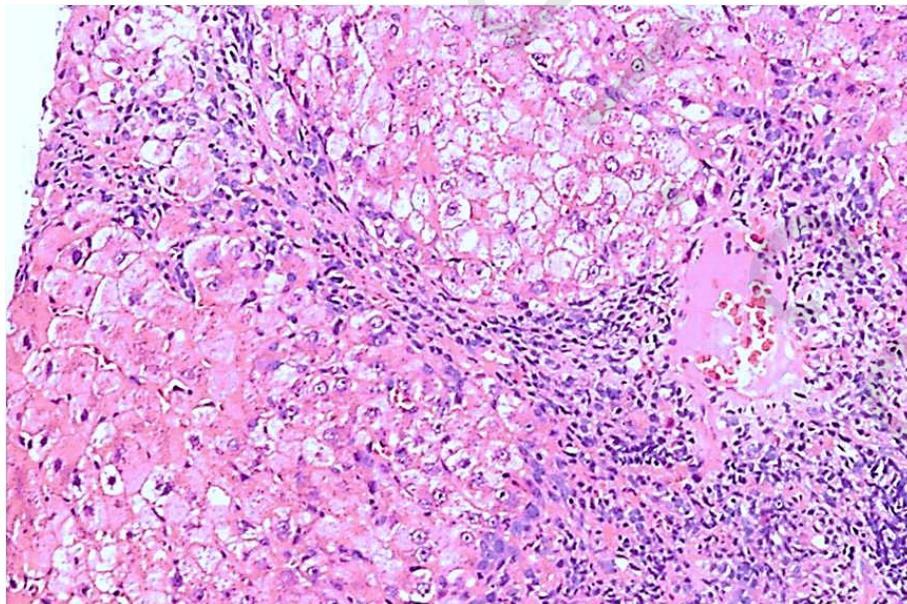
**Figure 14: A core liver biopsy from a patient with chronic hepatitis C, METAVIR A2, F2 (H&E stain x200).**



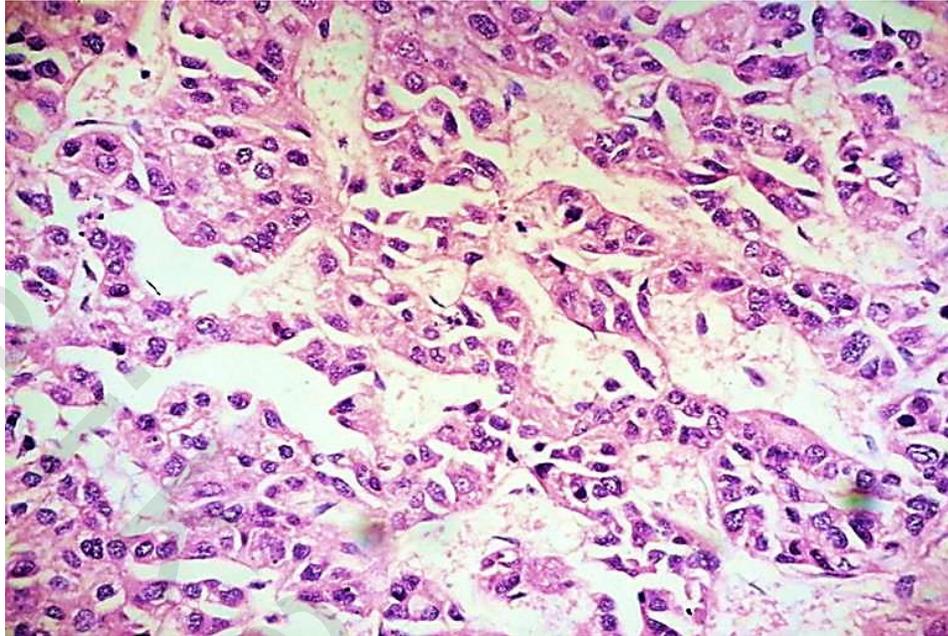
**Figure 15: A core liver biopsy from a patient with chronic hepatitis C, METAVIR A1, F2 with marked steatosis (H&E stain x100).**



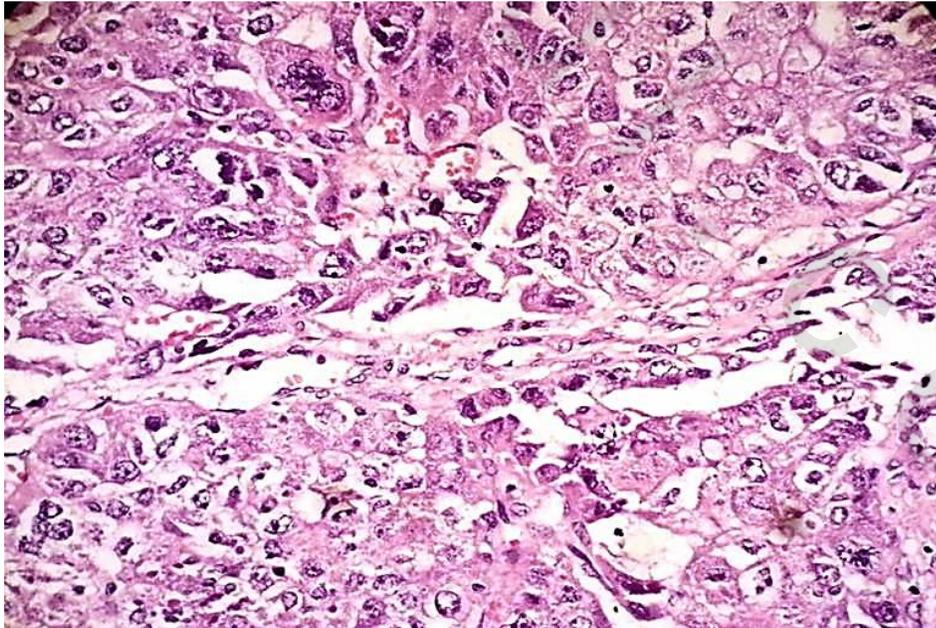
**Figure 16: A core liver biopsy from a patient with chronic hepatitis C, METAVIR A3, F3 with marked steatosis (H&E stain x100).**



**Figure 17: A high power view of the previous patient (H&E x200).**



**Figure 18: Hepatocellular carcinoma, trabecular pattern, grade 3, (H&E stain) (x200).**



**Figure 19: Hepatocellular carcinoma, solid pattern, grade 4, (H&E stain) (x400).**

**Table XIX: Immunohistochemical expression of mammalian target of rapamycin (mTOR) and autophagy-related protein 5 (Atg5) in liver biopsies of patients with chronic hepatitis C virus (HCV) infection.**

| Patient No. | mTOR expression    |                       | Atg5 expression    |              |
|-------------|--------------------|-----------------------|--------------------|--------------|
|             | Staining intensity | Localization          | Staining intensity | Localization |
| 1           | 1                  | Nuclear               | 3                  | Cytoplasmic  |
| 2           | 0                  | -                     | 3                  | Cytoplasmic  |
| 3           | 3                  | Cytoplasmic           | 2                  | Cytoplasmic  |
| 4           | 2                  | Cytoplasmic + Nuclear | 3                  | Cytoplasmic  |
| 5           | 0                  | -                     | 3                  | Cytoplasmic  |
| 6           | 2                  | Nuclear               | 0                  | -            |
| 7           | 0                  | -                     | 3                  | Cytoplasmic  |
| 8           | 3                  | Nuclear               | 1                  | Cytoplasmic  |
| 9           | 0                  | -                     | 3                  | Cytoplasmic  |
| 10          | 3                  | Nuclear               | 2                  | Cytoplasmic  |
| 11          | 3                  | Nuclear               | 2                  | Cytoplasmic  |
| 12          | 0                  | -                     | 3                  | Cytoplasmic  |
| 13          | 0                  | -                     | 3                  | Cytoplasmic  |
| 14          | 0                  | -                     | 3                  | Cytoplasmic  |
| 15          | 3                  | Cytoplasmic           | 2                  | Cytoplasmic  |
| 16          | 0                  | -                     | 3                  | Cytoplasmic  |
| 17          | 2                  | Nuclear               | 0                  | -            |
| 18          | 1                  | Cytoplasmic           | 2                  | Cytoplasmic  |
| 19          | 1                  | Nuclear               | 3                  | Cytoplasmic  |
| 20          | 3                  | Cytoplasmic           | 2                  | Cytoplasmic  |
| 21          | 3                  | Cytoplasmic + Nuclear | 2                  | Cytoplasmic  |
| 22          | 3                  | Cytoplasmic + Nuclear | 2                  | Cytoplasmic  |
| 23          | 0                  | -                     | 2                  | Cytoplasmic  |
| 24          | 0                  | -                     | 2                  | Cytoplasmic  |
| 25          | 0                  | -                     | 3                  | Cytoplasmic  |
| 26          | 2                  | Cytoplasmic + Nuclear | 3                  | Cytoplasmic  |
| 27          | 0                  | -                     | 3                  | Cytoplasmic  |
| 28          | 2                  | Cytoplasmic + Nuclear | 3                  | Cytoplasmic  |
| 29          | 2                  | Cytoplasmic           | 3                  | Cytoplasmic  |
| 30          | 1                  | Nuclear               | 3                  | Cytoplasmic  |

**Table XX: Immunohistochemical expression of mammalian target of rapamycin (mTOR) and autophagy-related protein 5 (Atg5) in HCV-related hepatocellular carcinoma (HCC) and the surrounding non-neoplastic liver tissues.**

| Patient No | HCV-related HCC    |                       |                    |              | Surrounding non-neoplastic liver |              |                    |              |
|------------|--------------------|-----------------------|--------------------|--------------|----------------------------------|--------------|--------------------|--------------|
|            | mTOR expression    |                       | Atg5 expression    |              | mTOR expression                  |              | Atg5 expression    |              |
|            | Staining intensity | Localization          | Staining intensity | Localization | Staining intensity               | Localization | Staining intensity | Localization |
| 1          | 3                  | Nuclear               | 1                  | Cytoplasmic  | 2                                | Cytoplasmic  | 3                  | Cytoplasmic  |
| 2          | 3                  | Nuclear               | 2                  | Cytoplasmic  | 1                                | Nuclear      | 3                  | Cytoplasmic  |
| 3          | 3                  | Cytoplasmic + Nuclear | 2                  | Cytoplasmic  | 3                                | Cytoplasmic  | 2                  | Cytoplasmic  |
| 4          | 3                  | Cytoplasmic           | 1                  | Cytoplasmic  | 2                                | Cytoplasmic  | 3                  | Cytoplasmic  |
| 5          | 1                  | Cytoplasmic           | 3                  | Cytoplasmic  | 3                                | Cytoplasmic  | 2                  | Cytoplasmic  |
| 6          | 3                  | Nuclear               | 1                  | Cytoplasmic  | 2                                | Nuclear      | 2                  | Cytoplasmic  |
| 7          | 0                  | -                     | 3                  | Cytoplasmic  | 0                                | -            | 2                  | Cytoplasmic  |
| 8          | 3                  | Cytoplasmic + Nuclear | 1                  | Cytoplasmic  | 3                                | Cytoplasmic  | 3                  | Cytoplasmic  |
| 9          | 3                  | Nuclear               | 2                  | Cytoplasmic  | 3                                | Cytoplasmic  | 2                  | Cytoplasmic  |
| 10         | 3                  | Nuclear               | 2                  | Cytoplasmic  | 1                                | Nuclear      | 3                  | Cytoplasmic  |
| 11         | 0                  | -                     | 3                  | Cytoplasmic  | 0                                | -            | 3                  | Cytoplasmic  |
| 12         | 3                  | Cytoplasmic           | 2                  | Cytoplasmic  | 2                                | Cytoplasmic  | 2                  | Cytoplasmic  |
| 13         | 3                  | Cytoplasmic           | 1                  | Cytoplasmic  | 1                                | Cytoplasmic  | 3                  | Cytoplasmic  |
| 14         | 2                  | Cytoplasmic           | 3                  | Cytoplasmic  | 2                                | Cytoplasmic  | 3                  | Cytoplasmic  |

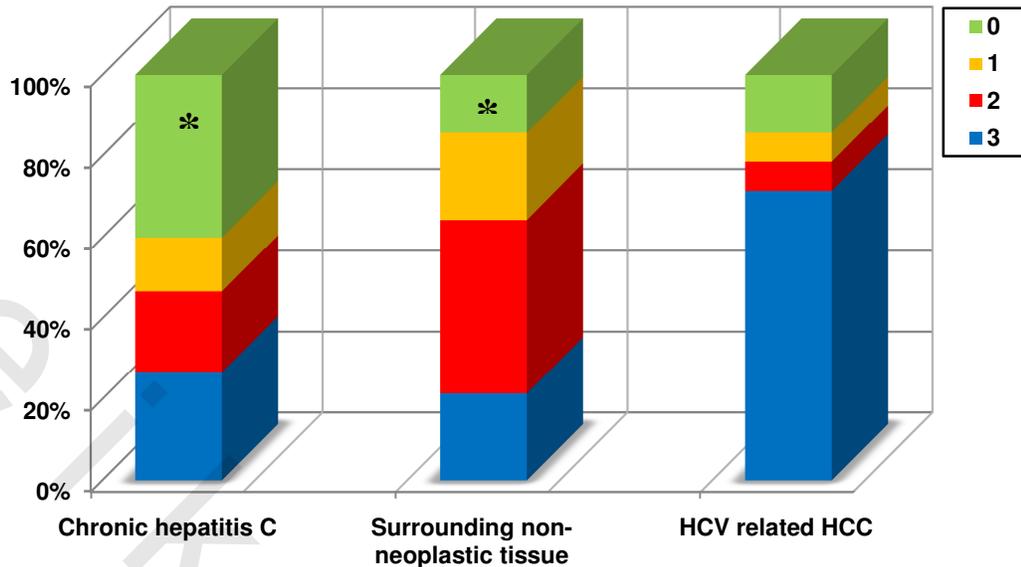
**Table XXI: Distribution of immunostaining positivity of mammalian target of rapamycin (mTOR) and autophagy-related protein 5 (Atg5) in chronic hepatitis C virus (HCV) infection, HCV-related hepatocellular carcinoma (HCC) and the surrounding non-neoplastic liver tissues.**

| Parameters                    | Chronic HCV infection (n = 30) | HCV-related HCC (n = 14) | Surrounding non-neoplastic liver (n = 14) | FET                  | P value |
|-------------------------------|--------------------------------|--------------------------|---|----------------------|---------|
| <b>mTOR expression:</b>       |                                |                          |   |                      |         |
| <b>Staining intensity:</b>    |                                |                          |   |                      |         |
| - 0 (%)                       | 12 (40.0)                      | 2 (14.3)                 | 2 (14.3)                                  | 13.096 <sup>ab</sup> | 0.032   |
| - 1 (%)                       | 4 (13.3)                       | 1 (7.1)                  | 3 (21.4)                                  |                      |         |
| - 2 (%)                       | 6 (20.0)                       | 1 (7.1)                  | 6 (42.9)                                  |                      |         |
| - 3 (%)                       | 8 (26.7)                       | 10 (71.4)                | 3 (21.4)                                  |                      |         |
| <b>Localization:</b>          | n = 18                         | n = 12                   | n = 12                                    | 0.849                | 0.725   |
| - Cytoplasmic (%)             | 5 (27.8)                       | 5 (41.7)                 | 9 (75.0)                                  |                      |         |
| - Nuclear (%)                 | 8 (44.4)                       | 5 (41.7)                 | 3 (25.0)                                  |                      |         |
| - Cytoplasmic and nuclear (%) | 5 (27.8)                       | 2 (16.6)                 | 0 (0.0)                                   |                      |         |
| <b>Atg5 expression</b>        |                                |                          |   |                      |         |
| <b>Staining intensity</b>     |                                |                          |   |                      |         |
| - 0 (%)                       | 2 (6.7)                        | 0 (0.0)                  | 0 (0.0)                                   | 11.297 <sup>ab</sup> | 0.038   |
| - 1 (%)                       | 1 (3.3)                        | 5 (35.7)                 | 0 (0.0)                                   |                      |         |
| - 2 (%)                       | 10 (33.3)                      | 5 (35.7)                 | 6 (42.9)                                  |                      |         |
| - 3 (%)                       | 17 (56.7)                      | 4 (28.6)                 | 8 (57.1)                                  |                      |         |
| <b>Localization:</b>          | n = 28                         |                          |   |                      |         |
| - Cytoplasmic (%)             | 28 (100.0)                     | 14 (100.0)               | 14 (100.0)                                | -                    | -       |

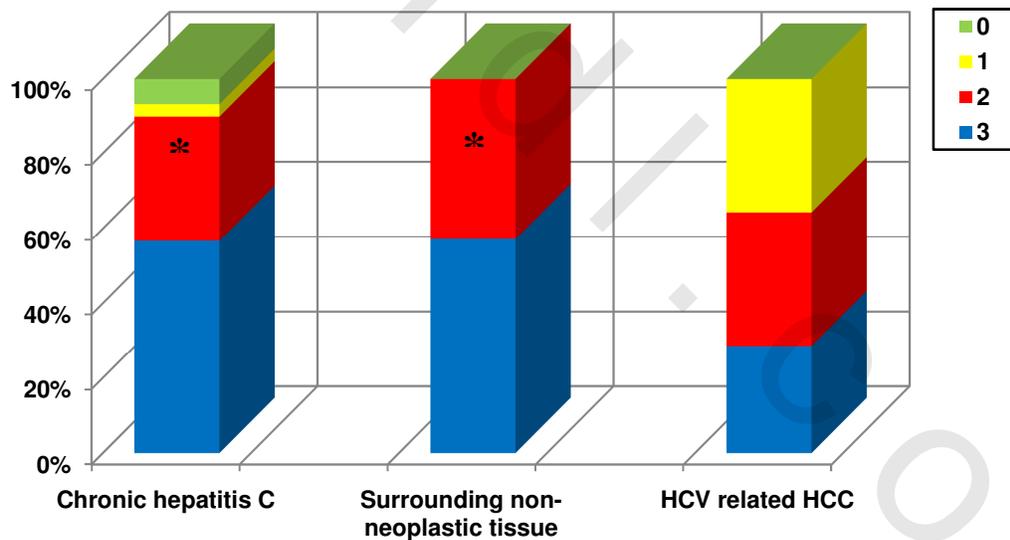
FET = Fisher's Exact test.

<sup>a</sup> = Significant statistical difference between HCV-related HCC and chronic HCV infection tissues.

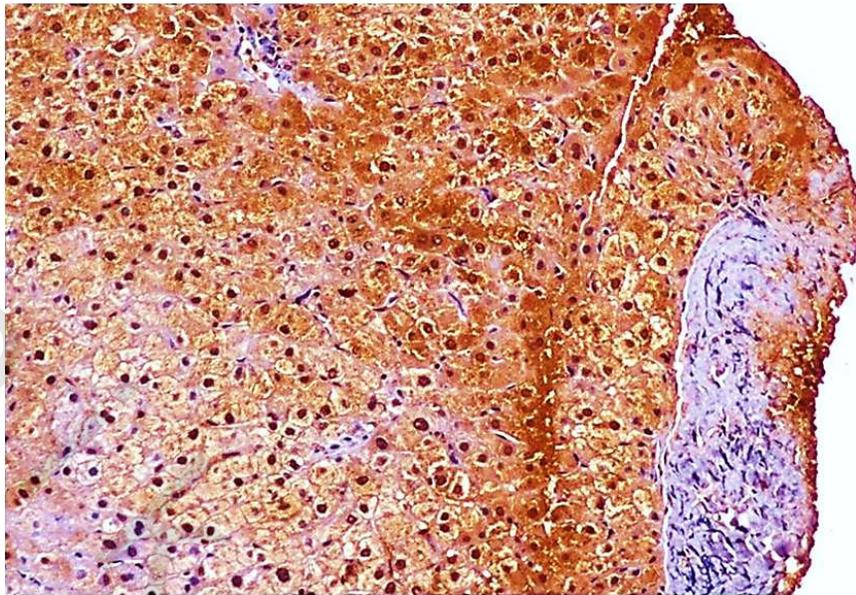
<sup>b</sup> = Significant statistical difference between HCV-related HCC and the surrounding non-neoplastic liver tissues.



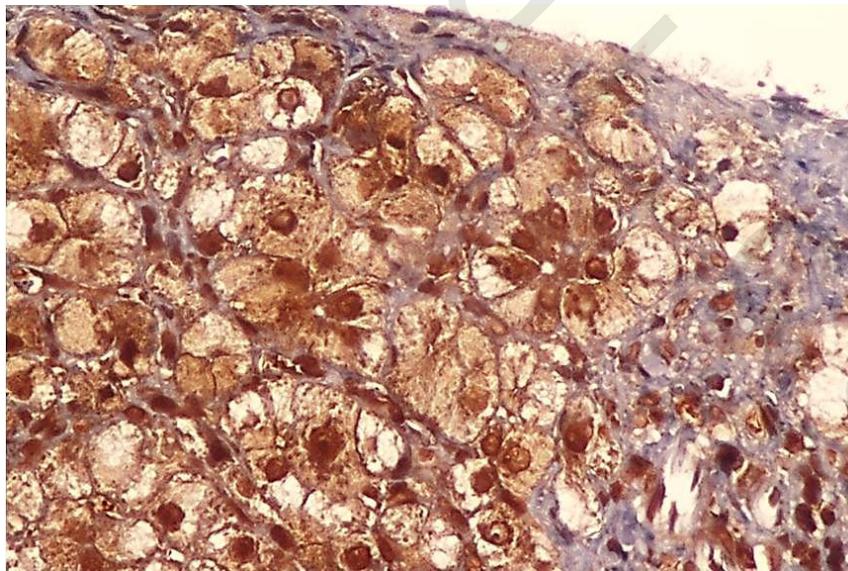
**Figure 20: Statistical comparisons between chronic hepatitis C virus (HCV) infection, HCV-related hepatocellular carcinoma (HCC) and the surrounding non-neoplastic liver tissues as regards mammalian target of rapamycin (mTOR) staining intensity ( $P = 0.032$ ). \* = Significant statistical difference from HCV-related HCC tissues.**



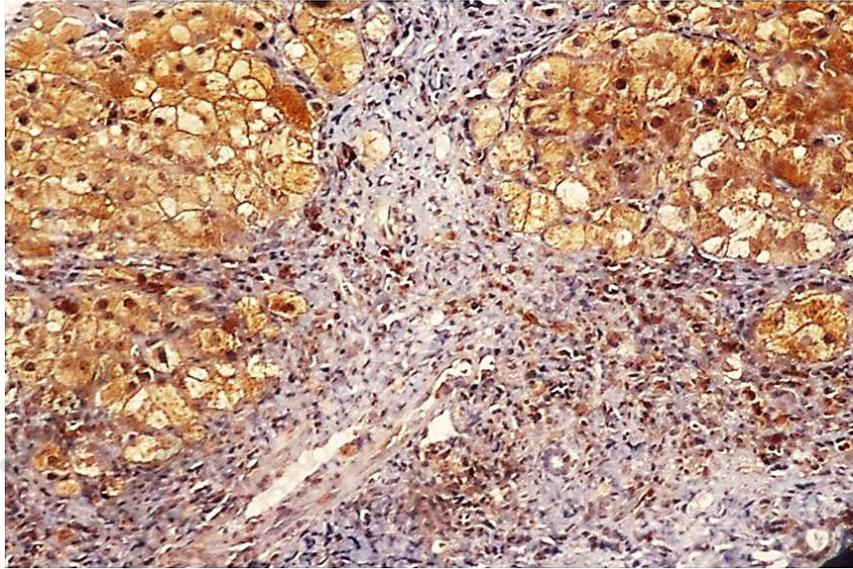
**Figure 21: Statistical comparisons between chronic hepatitis C virus (HCV) infection, HCV-related hepatocellular carcinoma (HCC) and the surrounding non-neoplastic liver tissues as regards autophagy-related protein 5 (Atg5) staining intensity ( $P = 0.038$ ). \* = Significant statistical difference from HCV-related HCC tissues.**



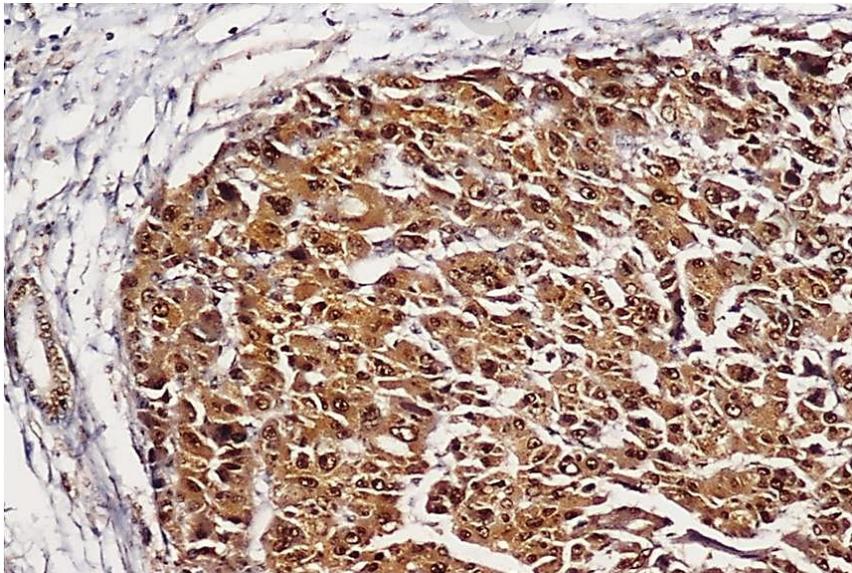
**Figure 22:** A core liver biopsy from a patient with chronic hepatitis C, METAVIR F2 showing moderate cytoplasmic staining of mammalian target of rapamycin (mTOR) antibody (HRP/DAB x200).



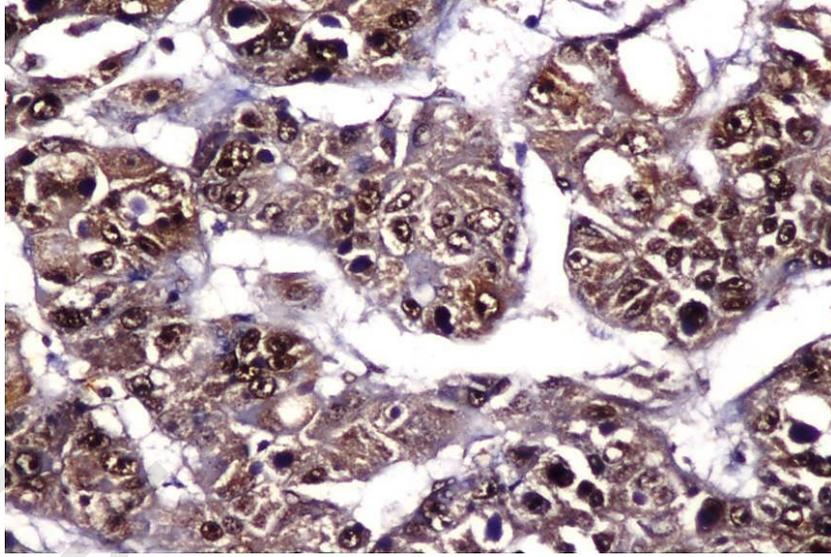
**Figure 23:** A core liver biopsy from a patient with chronic hepatitis C with rosette formation showing strong cytoplasmic and nuclear stain of mammalian target of rapamycin (mTOR) antibody (HRP/DAB x400).



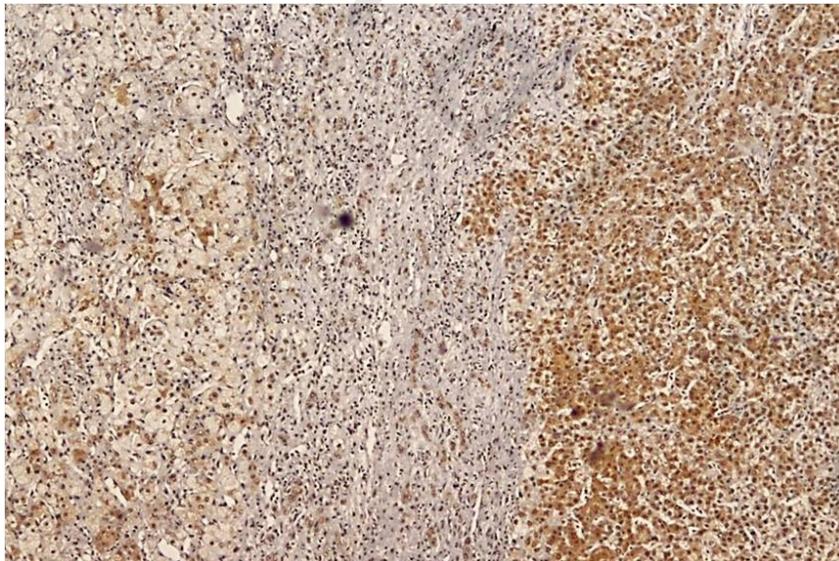
**Figure 24: A core liver biopsy from a patient with HCV-related cirrhosis, METAVIR F4, showing strong diffuse cytoplasmic stain of mammalian target of rapamycin (mTOR) antibody (HRP/DAB x200).**



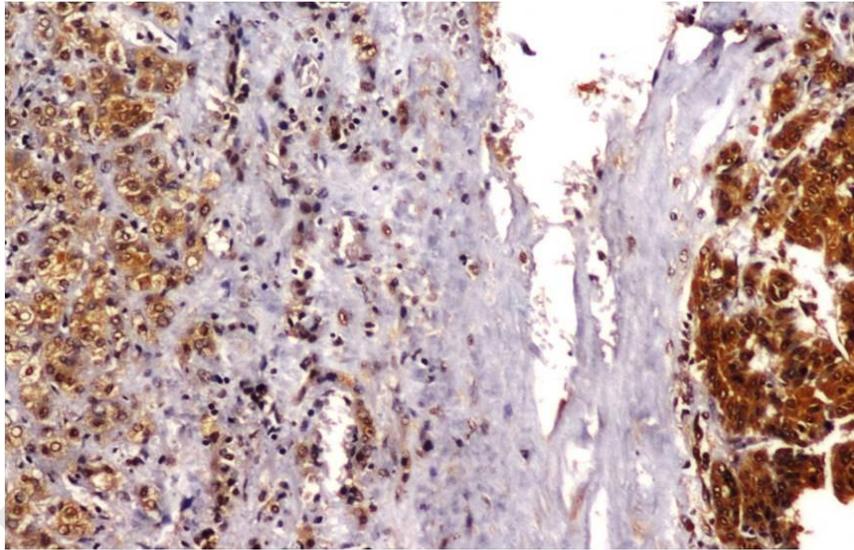
**Figure 25: Hepatocellular carcinoma, Grade 4, showing strong cytoplasmic staining of mammalian target of rapamycin (mTOR) antibody (HRP/DAB x200).**



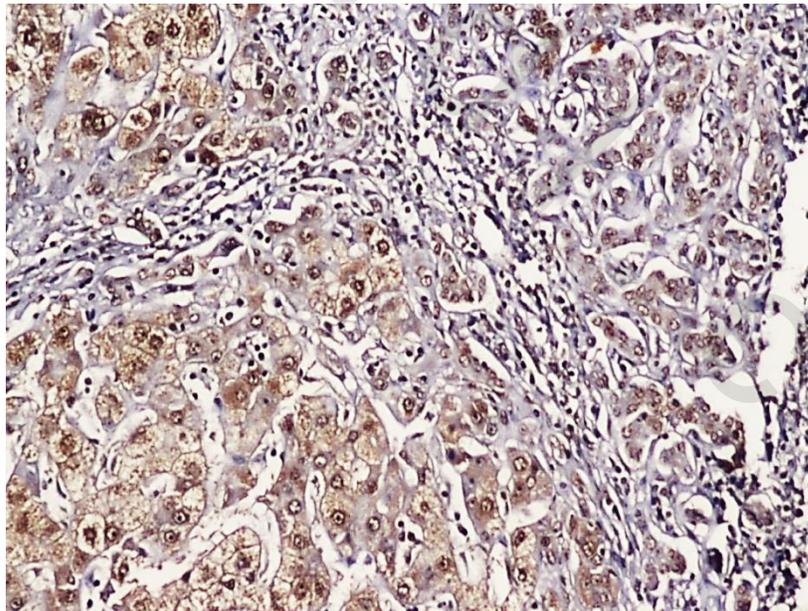
**Figure 26: Hepatocellular carcinoma, Grade 3, trabecular pattern showing strong cytoplasmic and nuclear staining of mammalian target of rapamycin (mTOR) antibody (HRP/DAB x400).**



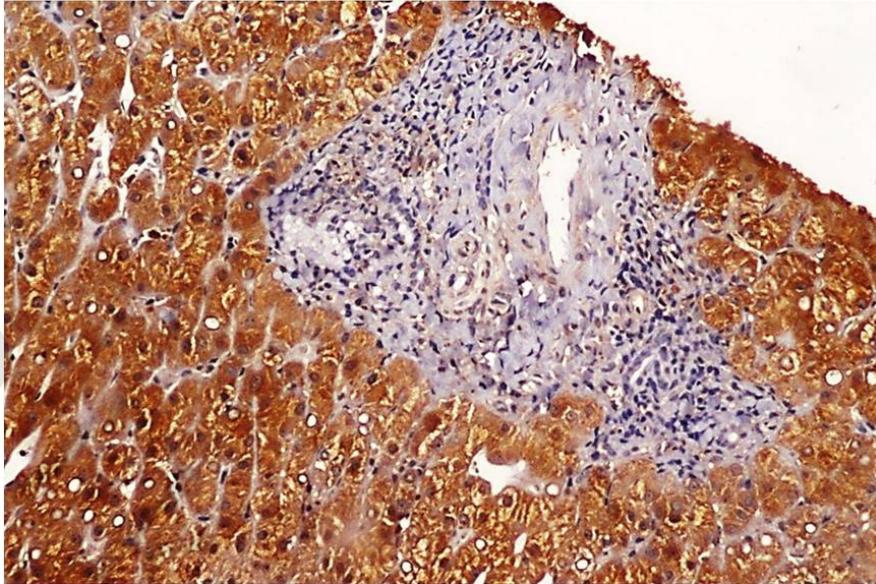
**Figure 27: Hepatocellular carcinoma, Grade 2 showing strong cytoplasmic staining of mammalian target of rapamycin (mTOR) antibody and less intense stain in the surrounding non-neoplastic tissue. Notice the difference in staining pattern from the neoplastic tissue on the right and non-neoplastic tissue on the left (HRP/DAB x100).**



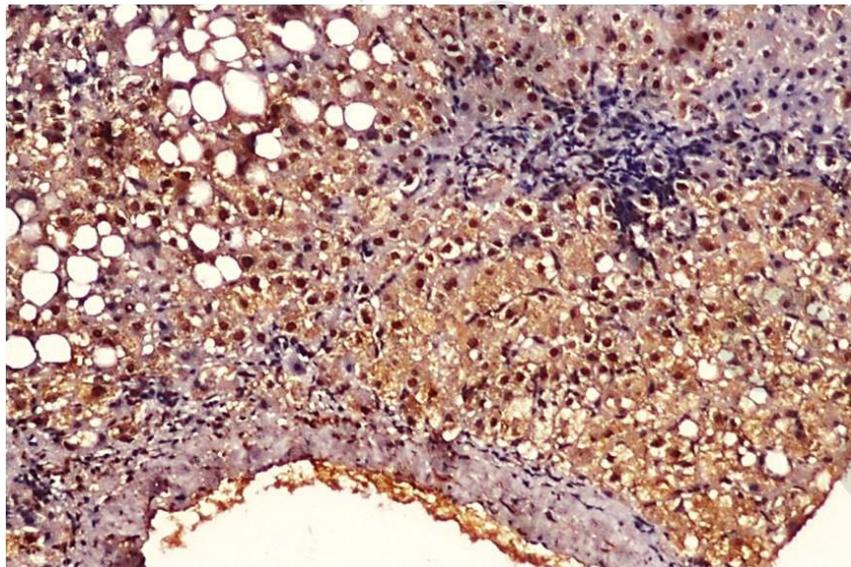
**Figure 28: Hepatocellular carcinoma, Grade 3, showing strong staining of mammalian target of rapamycin (mTOR) in tumor tissue (right) versus weak mTOR staining in the surrounding non neoplastic tissue (left) (HRP/DAB x200).**



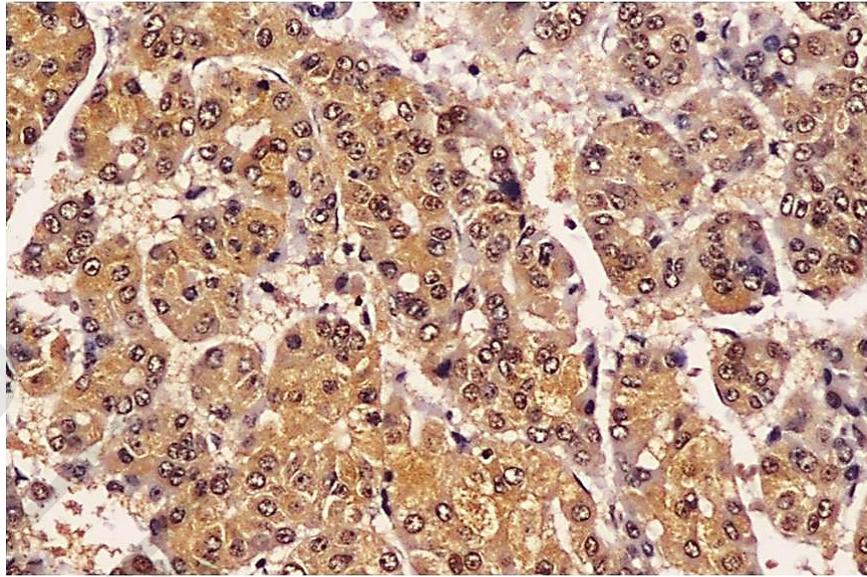
**Figure 29: Hepatocellular carcinoma, Grade 2, showing weak cytoplasmic stain of mammalian target of rapamycin (mTOR) (HRP/DAB x200).**



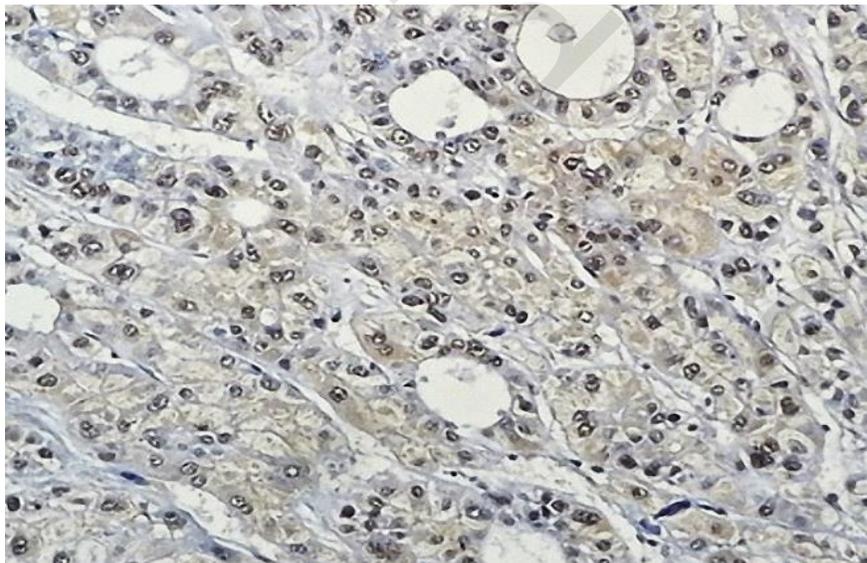
**Figure 30: A core liver biopsy from a patient with chronic hepatitis C, METAVIR F2, showing strong cytoplasmic staining of autophagy-related protein 5 (Atg5) antibody (HRP/DAB x200).**



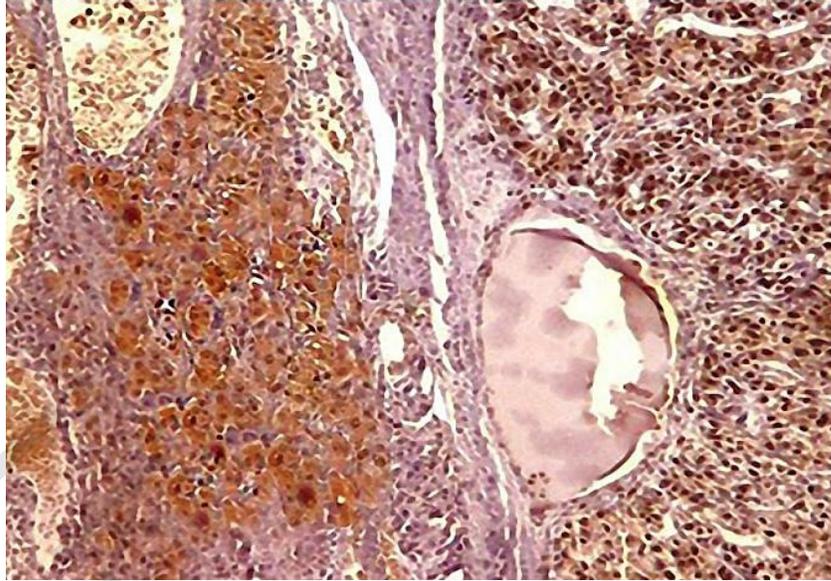
**Figure 31: A core liver biopsy from a patient with chronic hepatitis C, METAVIR A2 F2, with moderate steatosis showing strong cytoplasmic staining of autophagy-related protein 5 (Atg5) antibody (HRP/DAB x200).**



**Figure 32: Hepatocellular carcinoma, Grade 2, showing strong cytoplasmic staining of autophagy-related protein 5 (Atg5) antibody (HRP/DAB x400).**



**Figure 33: Hepatocellular carcinoma, Grade 2, showing weak cytoplasmic staining of autophagy-related protein 5 (Atg5) antibody (HRP/DAB x200).**



**Figure 34: Hepatocellular carcinoma showing weak cytoplasmic staining for autophagy-related protein 5 (Atg5) antibody (right) versus strong Atg5 staining in the surrounding non-neoplastic tissue (left) (HRP/DAB x400).**

**Table XXII: Statistical correlations (“r” value) between serum mammalian target of rapamycin (mTOR) levels, intrahepatic expression of mTOR and autophagy-related protein 5 (Atg5) and other parameters in patients with chronic hepatitis C virus (HCV) infection.**

| Parameters                                | Serum mTOR<br>(ng/ml)<br>(n = 40) |            | mTOR<br>expression<br>(n = 30) |            | Atg5<br>expression<br>(n = 30) |            |
|---|-----------------------------------|------------|--------------------------------|------------|--------------------------------|------------|
|   | r<br>value                        | P<br>value | r*<br>value                    | P<br>value | r*<br>value                    | P<br>value |
| Age (years)                               | 0.194                             | 0.232      | 0.029                          | 0.880      | -0.045                         | 0.815      |
| Serum AST (U/L)                           | 0.547                             | < 0.001    | 0.555                          | 0.001      | -0.409                         | 0.025      |
| Serum ALT (U/L)                           | 0.624                             | < 0.001    | 0.501                          | 0.005      | -0.421                         | 0.021      |
| Serum GGT (U/L)                           | 0.232                             | 0.151      | 0.214                          | 0.255      | -0.017                         | 0.930      |
| Serum HCV RNA (x10 <sup>3</sup><br>IU/ml) | 0.091                             | 0.575      | -0.272                         | 0.146      | 0.324                          | 0.081      |
| METAVIR: (n = 30)                         |                                   |            |                                |            |                                |            |
| - Histological activity grade*            | 0.379                             | 0.039      | 0.442                          | 0.015      | -0.595                         | 0.001      |
| - Fibrosis stage*                         | 0.547                             | 0.002      | 0.489                          | 0.006      | -0.420                         | 0.021      |
| Steatosis grade*(n = 30)                  | 0.610                             | < 0.001    | 0.393                          | 0.032      | -0.190                         | 0.313      |
| mTOR expression*(n = 30)                  | 0.507                             | 0.004      | -                              | -          | -                              | -          |
| Atg5 expression*(n = 30)                  | -0.398                            | 0.029      | -0.611                         | < 0.001    | -                              | -          |

AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; GGT = Gamma glutamyltranspeptidase.

\* Spearman rho correlation

**Table XXIII: Statistical correlations (“r” value) between serum mammalian target of rapamycin (mTOR) levels, intratumoral expression of mTOR and autophagy-related protein 5 (Atg5) and other parameters in patients with hepatitis C virus-related hepatocellular carcinoma (HCC) (n = 14).**

| Parameters                               | Serum mTOR (ng/ml) |         | mTOR expression |         | Atg5 expression |         |
|--|--------------------|---------|-----------------|---------|-----------------|---------|
|  | r value            | P value | r* value        | P value | r* value        | P value |
| Serum AFP (ng/ml)                        | 0.725              | 0.003   | 0.728           | 0.003   | -0.713          | 0.004   |
| Child-Pugh score*                        | 0.110              | 0.707   | -0.048          | 0.870   | -0.107          | 0.717   |
| MELD score*                              | 0.297              | 0.303   | 0.136           | 0.643   | -0.199          | 0.495   |
| HCC maximum diameter (cm)                | 0.618              | 0.018   | 0.574           | 0.032   | -0.840          | <0.001  |
| CLIP stage*                              | 0.573              | 0.032   | 0.762           | 0.002   | -0.836          | < 0.001 |
| HCC grade*                               | 0.736              | 0.003   | 0.593           | 0.025   | -0.808          | < 0.001 |
| mTOR expression*                         | 0.571              | 0.033   | -               | -       | -               | -       |
| Atg5 expression*                         | -0.703             | 0.005   | -0.818          | < 0.001 | -               | -       |
| Surrounding non-neoplastic liver tissue: |                    |         |                 |         |                 |         |
| - mTOR expression*                       | 0.119              | 0.684   | 0.318           | 0.268   | -0.134          | 0.649   |
| - Atg5 expression*                       | 0.395              | 0.162   | 0.112           | 0.702   | -0.266          | 0.359   |

AFP = Alpha fetoprotein; MELD = The Model for End Stage Liver Disease score; CLIP = The Cancer of the Liver Italian Program.

\* Spearman rho correlation

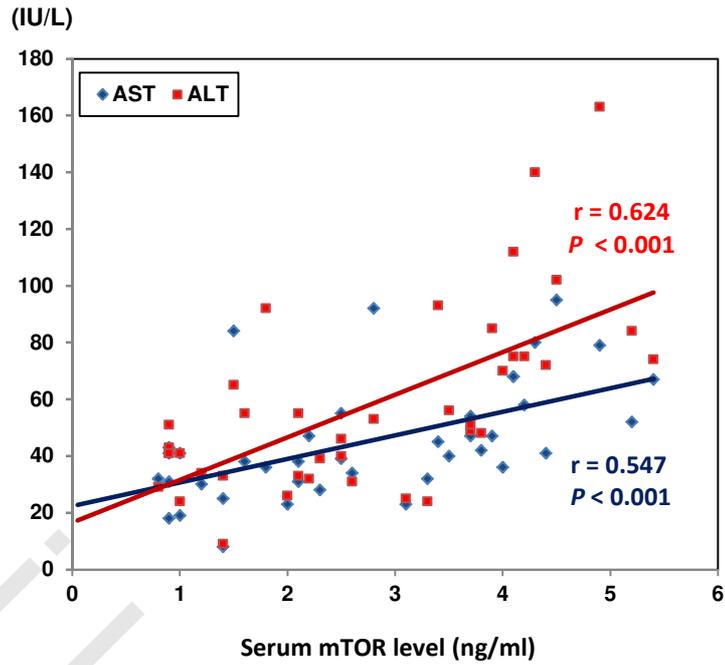


Figure 35: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in patients with chronic hepatitis C virus infection ( $P < 0.001$ ).

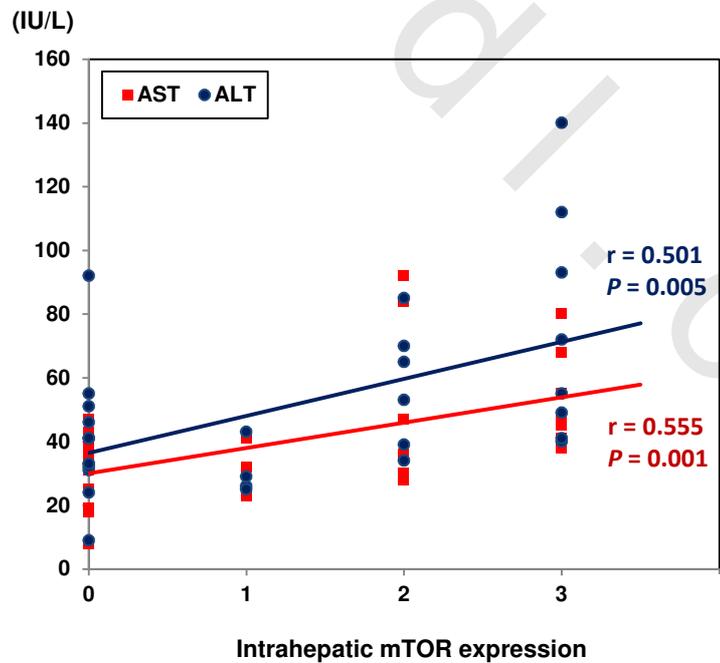


Figure 36: Statistical correlation between intrahepatic mammalian target of rapamycin (mTOR) expression and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in patients with chronic hepatitis C virus infection ( $P = 0.001$  and  $P = 0.005$  respectively).

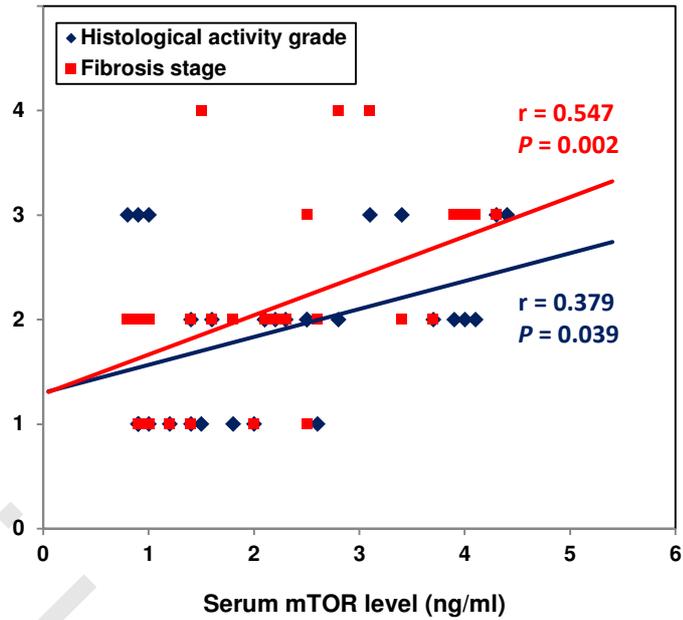


Figure 37: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels and METAVIR histological activity grade and fibrosis stage in patients with chronic hepatitis C virus infection ( $P = 0.039$  and  $P = 0.002$  respectively).

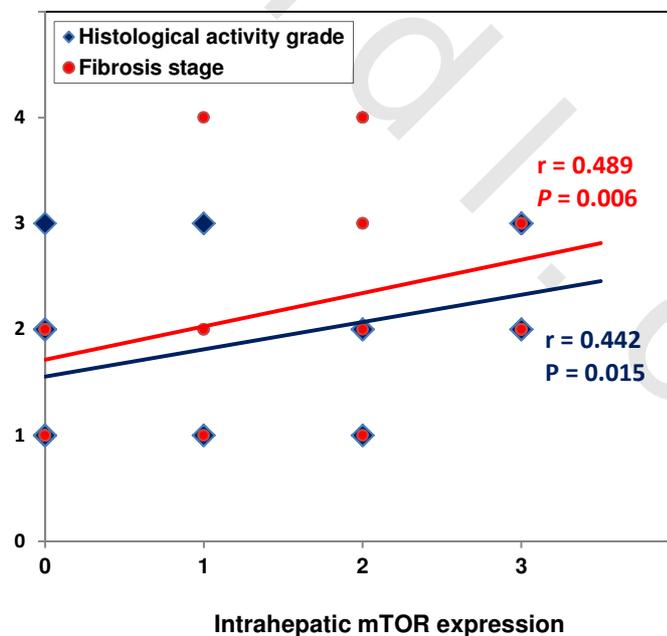
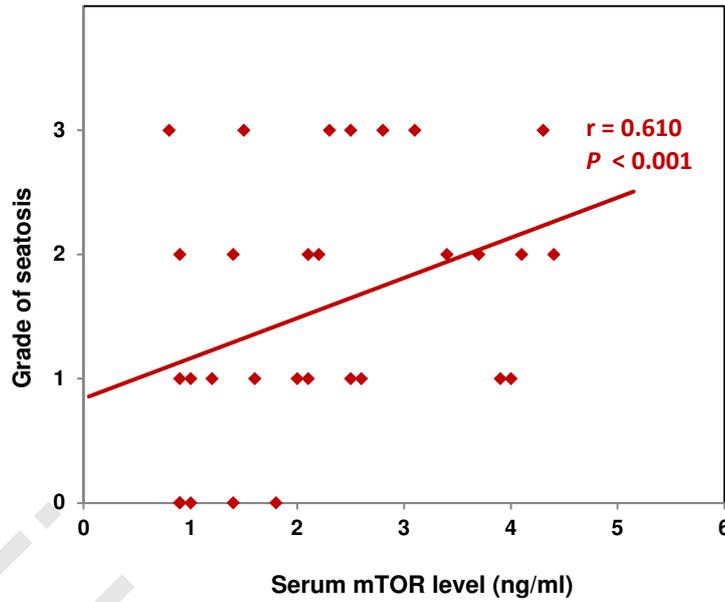
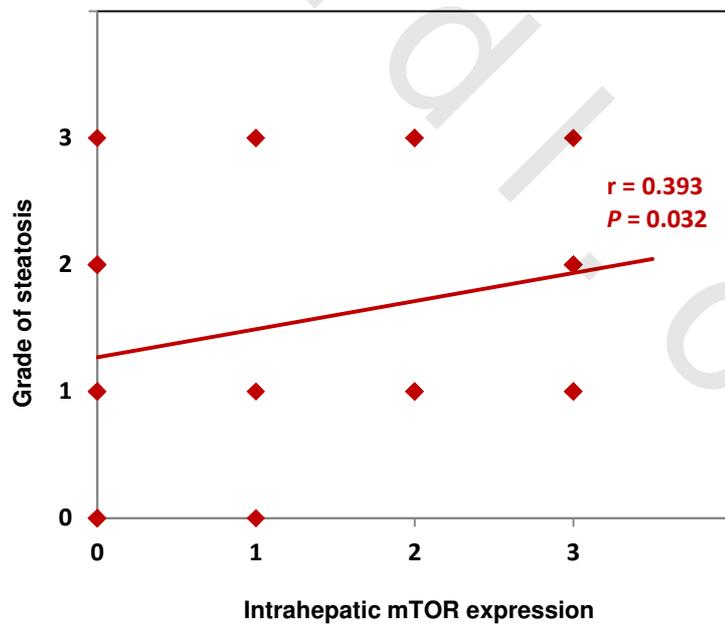


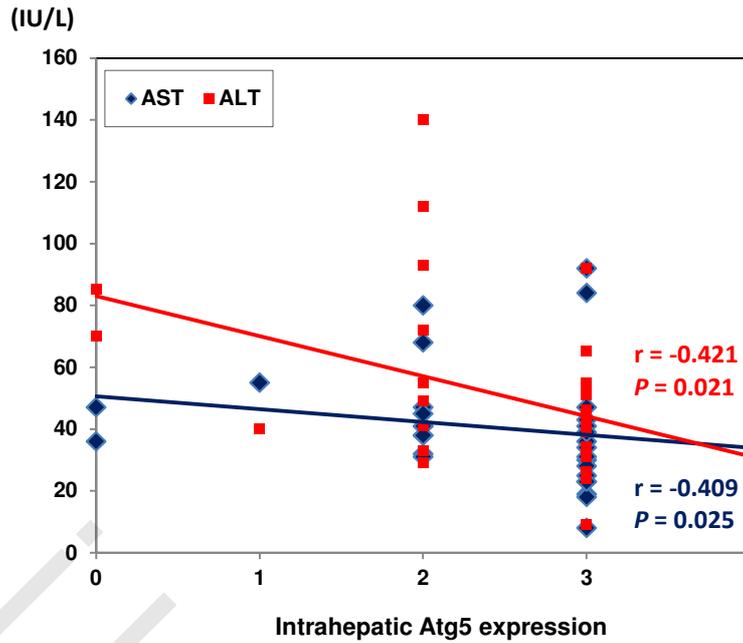
Figure 38: Statistical correlation between intrahepatic mammalian target of rapamycin (mTOR) expression and METAVIR histological activity grade and fibrosis stage in patients with chronic hepatitis C virus infection ( $P = 0.015$  and  $P = 0.006$  respectively).



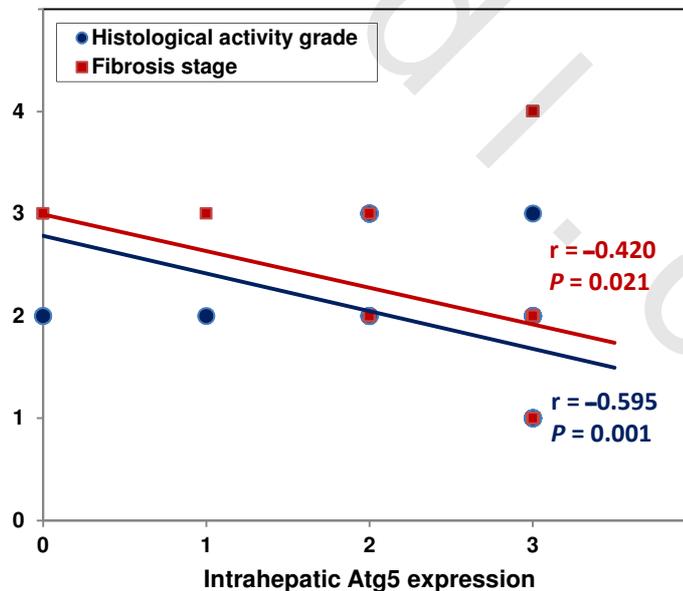
**Figure 39: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels and steatosis grade in patients with chronic hepatitis C virus infection ( $P < 0.001$ ).**



**Figure 40: Statistical correlation between intrahepatic mammalian target of rapamycin (mTOR) expression and steatosis grade in patients with chronic hepatitis C virus infection ( $P = 0.032$ ).**



**Figure 41:** Statistical correlation between intrahepatic autophagy-related protein 5 (Atg5) expression and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in patients with chronic hepatitis C virus infection ( $P = 0.025$  and  $P = 0.021$  respectively).



**Figure 42:** Statistical correlation between intrahepatic autophagy-related protein 5 (Atg5) expression and METAVIR histological activity grade and fibrosis stage in patients with chronic hepatitis C virus infection ( $P = 0.001$  and  $P = 0.021$  respectively).

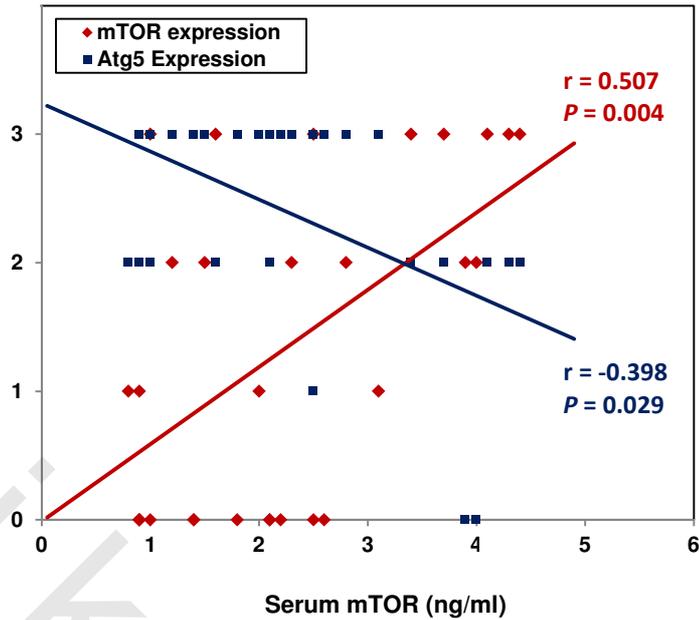


Figure 43: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels on one hand and intrahepatic mTOR expression and autophagy-related protein 5 (Atg5) on the other hand in patients with chronic hepatitis C virus infection ( $P = 0.004$  and  $P = 0.029$  respectively).

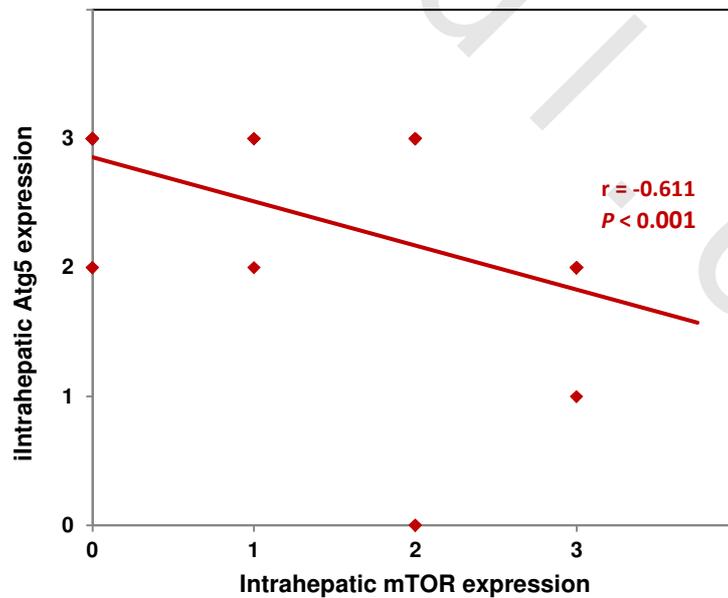


Figure 44: Statistical correlation between expression of mTOR and autophagy-related protein 5 (Atg5) in patients with chronic hepatitis C virus infection ( $P < 0.001$ ).

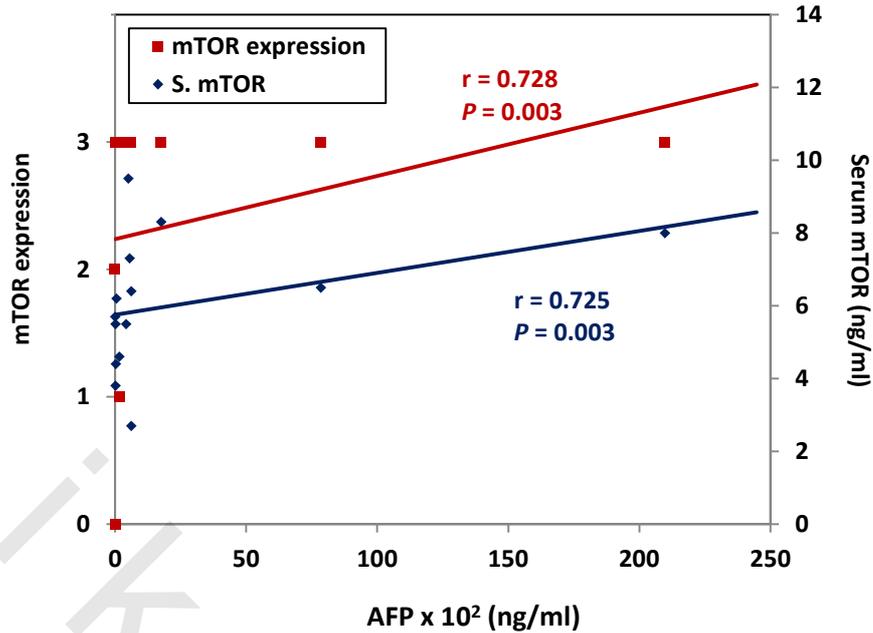


Figure 45: Statistical correlations between serum mammalian target of rapamycin (mTOR) levels and intra-tumoral mTOR expression on one hand and serum alpha fetoprotein (AFP) levels on the other hand in patients with hepatitis C virus-related hepatocellular carcinoma ( $P = 0.003$ ).

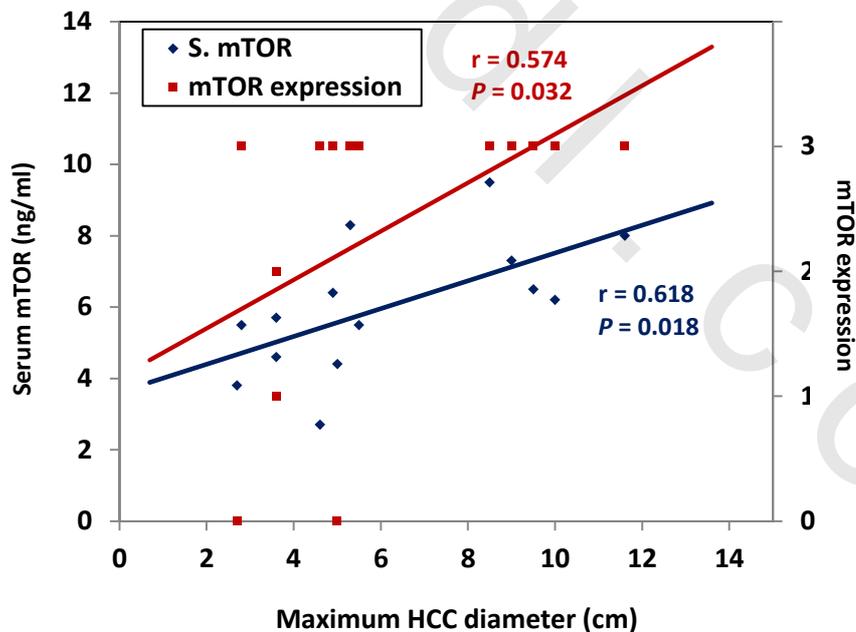


Figure 46: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels and intra-tumoral mTOR expression on one hand and tumor maximum diameter on the other hand in patients with hepatitis C virus-related hepatocellular carcinoma ( $P = 0.018$  and  $P = 0.032$  respectively).

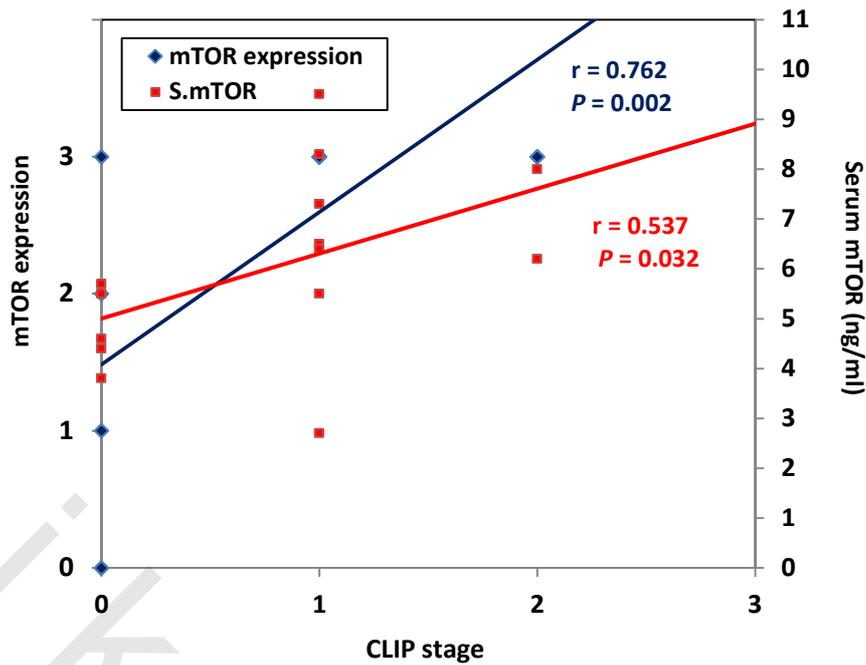


Figure 47: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels and intra-tumoral mTOR expression on one hand and the Cancer of the Liver Italian Program (CLIP) stage on the other hand in patients with hepatitis C virus-related hepatocellular carcinoma ( $P = 0.032$  and  $P = 0.002$  respectively).

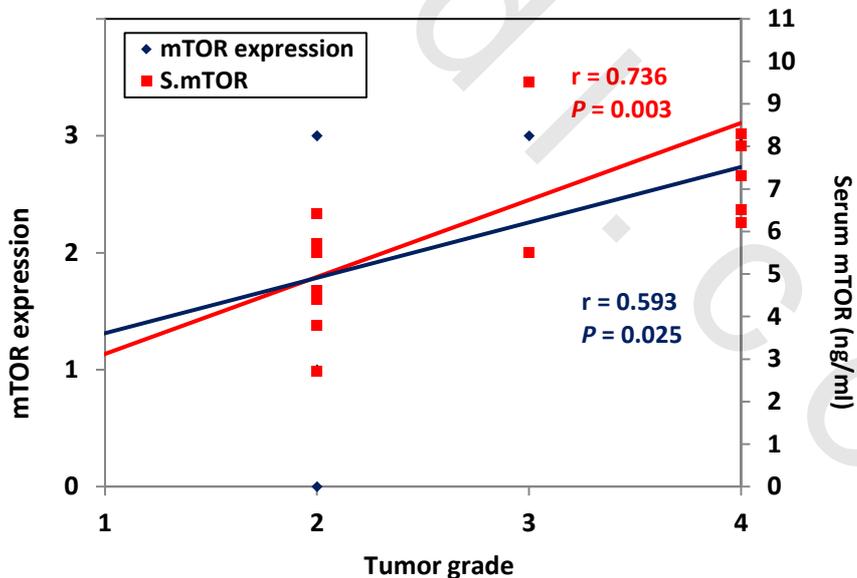
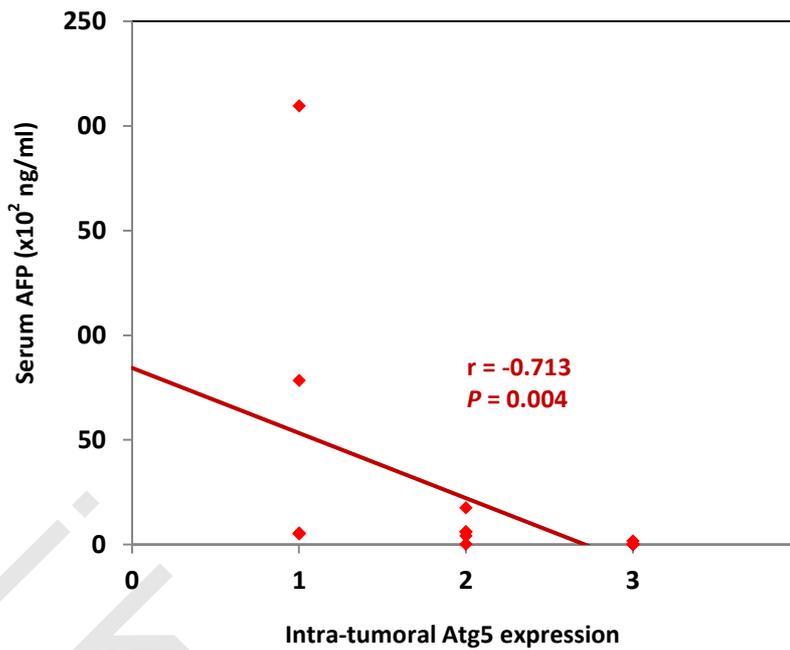
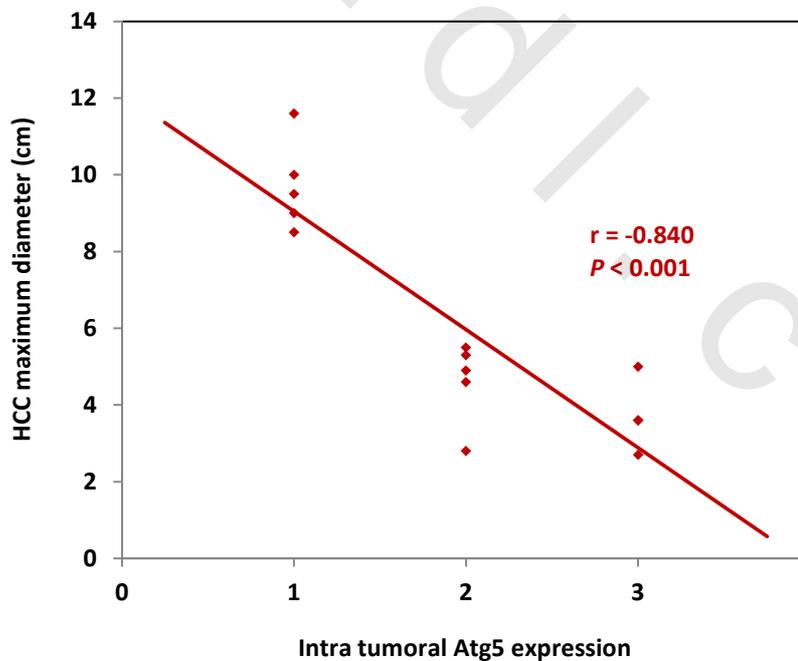


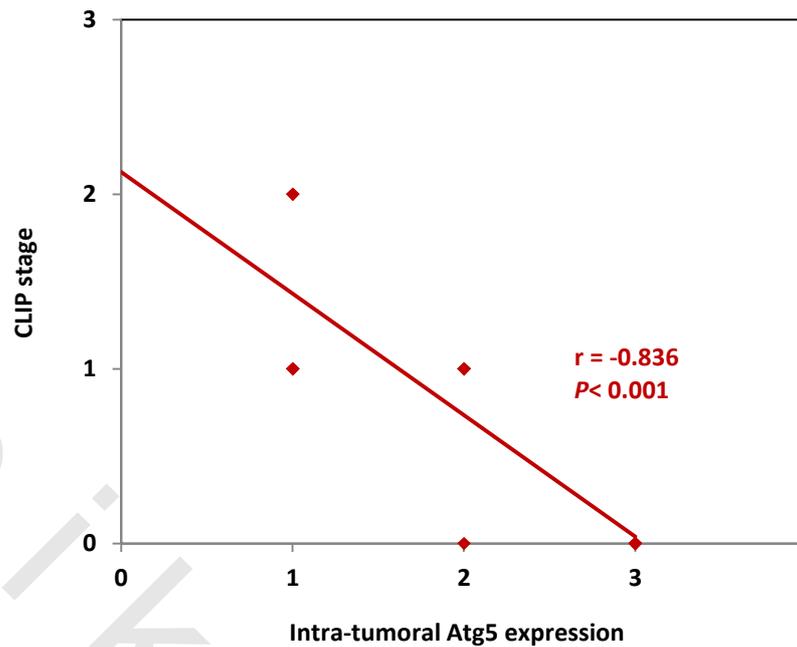
Figure 48: Statistical correlation between serum mammalian target of rapamycin (mTOR) levels and intra-tumoral mTOR expression on one hand and tumor histological grade on the other hand in patients with hepatitis C virus-related hepatocellular carcinoma ( $P = 0.003$  and  $P = 0.025$  respectively).



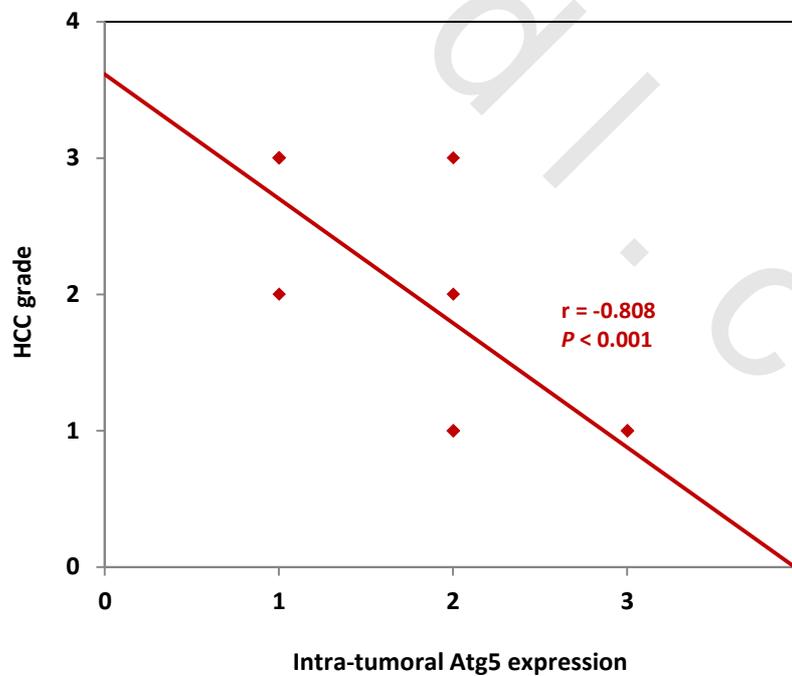
**Figure 49:** Statistical correlation between intra-tumoral autophagy-related protein 5 (Atg5) expression and serum alpha fetoprotein (AFP) levels in patients with hepatitis C virus-related hepatocellular carcinoma ( $P = 0.004$ ).



**Figure 50:** Statistical correlation between intra-tumoral autophagy-related protein 5 (Atg5) expression and tumor maximum diameter in patients with hepatitis C virus-related hepatocellular carcinoma ( $P < 0.001$ ).



**Figure 51: Statistical correlation between intra-tumoral autophagy-related protein 5 (Atg5) expression and the Cancer of the Liver Italian Program (CLIP) stage in patients with hepatitis C virus-related hepatocellular carcinoma ( $P < 0.001$ ).**



**Figure 52: Statistical correlation between intra-tumoral autophagy-related protein 5 (Atg5) expression and tumor histological grade in patients with hepatitis C virus-related hepatocellular carcinoma ( $P < 0.001$ ).**