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Anatomo-clinical correlations in hepatic steatosis in patients with C chronic hepatitis

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Abstract

Hepatic steatosis is a progressive liver disease, frequently met in chronic virus C hepatitis, playing an important role in its evolution towards fibrosis, necroinflammation and the final stage the hepatocellular carcinoma. The present paper studies the correlation between clinico-epidemiological parameters and the pathology test outcome in patients with hepatic biopsies carried out before they began the antiviral treatment. We used the classical histological staining and the immunolabeling. The presence of steatosis is not directly associated with clinico-epidemiological parameters and with the degree of fibrosis and inflammation.

Keywords: chronic hepatitis, hepatic steatosis, body mass index, fibrosis, inflammatory activity.

Introduction

Hepatitis C virus (HCV) is a major health problem worldwide, the number of people infected being estimated at 160–200 million worldwide [1–5]. Nearly one million people newly infected with HCV are registered around the world every year, and the majority of acute infections turn into chronic hepatitis [4]. Also, about 350 000 deaths worldwide caused by HCV infection are recorded annually [6, 7].

Chronic infection by HCV is associated with the development of insulin resistance, diabetes, and hepatic steatosis or cause more serious diseases such as cirrhosis and hepatocellular carcinoma [8–12].

Fatty liver is defined as a pathological condition characterized by the accumulation of triglycerides in the cytoplasm of hepatocytes in the form of micro- or macro-vesicles in less than 5% of hepatocytes [13]. Histopathological appearance of liver cells is a criterion for assessing the severity of steatosis injuries. Thus, depending on the number of hepatocytes affected, there is a classification of the severity of steatotic injuries: grade 0, when less than 5% of hepatocytes are affected; grade I (mild steatosis), when 6–33% of hepatocytes are affected; grade II (moderate steatosis), when 34–66% of hepatocytes are affected; grade III (severe steatosis), when over 66% of hepatocytes are affected [13, 14].

The relationship between chronic hepatitis C and steatosis is not fully elucidated, mechanisms of steatosis being extremely complex. It is believed that at the cellular level, an imbalance in the liver absorption of fatty, lipogenesis, β-oxidation and export of triglycerides in the form of particles of very low-density lipoproteins (VLDL) [13].

Aim

Our aim was to assess the histopathological aspects of hepatic steatosis in a group of patients with chronic hepatitis C admitted for clinical and histopathological evaluation for the establishment pegylated interferon therapy (pegylated interferon alpha 2) and ribavirin.

Patients, Materials and Methods

We have retrospectively studied a group of 52 patients with chronic C hepatitis admitted between April 2009–December 2014 at the "Filantropia" Municipal Hospital, Craiova, Romania, with a pathology and biologically confirmed diagnosis of chronic viral C hepatitis. We excluded from the study patients with known high intake of alcohol. All patients who were included were informed on their health state and signed the informed consent for participation in the study.

The epidemiological parameters used were age and gender and biological ones were aspartate transaminase (AST), alanine transaminase (ALT) and HCV viral load. The body mass index (BMI) was calculated using the formula: weight [kg] divided by squared height [m] and classified as: underweight <18.5; within normal range: 18.5–24.9; overweight: 25–29.9; grade I obesity: 30–34.9; grade II obesity: 35–39.9; grade III obesity: >40.

Liver biopsies were performed only before the

beginning of the antiviral treatment. Biopsy was performed by using the Menghini technique (Menghini Hepax Kit G16), with an intercostal approach on the anterior axillary line. Liver biopsy specimens were fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4 μm thickness and were stained with Hematoxylin–Eosin (HE) and the Green Light Goldner–Szeckeli (GS) trichrome. Hepatic fibrosis, inflammation and necrosis were assessed in relation to the HAI (Histological Activity Index) and Metavir scores [15].

For the immunohistochemical study, histological sections were collected on slides coated with poly-L-lysine and dried in a thermostat at 37^0C for 24 hours. Antigen unmasking was performed by boiling in a microwave oven in a sodium citrate solution (pH 6) for 21 minutes. Blocking endogenous peroxidase was done by incubating the slides in 3% hydrogen peroxide for 30 minutes, at room temperature, followed by washing in distilled water for 10 minutes and a wash in a solution of 1% phosphate-buffered saline (PBS) for 5 minutes. Blocking non-specific sites was achieved by incubating the slides in 2% skim milk for 30 minutes. Then, the sections were incubated with primary antibodies for 18 hours (overnight) in a refrigerator, at 40^0C. The next day, the biotinylated secondary antibody was applied for 30 minutes, at room temperature. The signal was detected using 3,3'-Diaminobenzidine (DAB) (Dako) and the reaction was stopped by washing the slides in a solution of 1% PBS. To contrast the slides, we used Mayer's Hematoxylin, and mounting the plates was carried out in DPX (Fluka) medium. We used the following markers to identify the inflammatory reaction occurring during steatosis: anti-CD3 (L26 clone, 1:100 dilution, Dako) for T-lymphocytes, anti-CD20 (L26 clone, 1:100 dilution, Dako) for T-lymphocytes, and anti-CD68 (KP1 clone, Dako, 1:200 dilution) for Kupffer cells and macrophages of the portal spaces.

Results

From the 52 patients included in the cohort, 19 (36.54%) were men and 33 (63.46%) were women. The male to female ratio was 1:1.74. The mean age was 46.48 years (ranging between 28–64 years). The average BMI was above 25 kg/m^2 in 19 cases. No patient presented a history or relevant laboratory data for diabetes (Table 1).

Table 1 – ***Clinical data of the studied group***

No. of patients with chronic HCV (*n*)	52
Mean age [years]	46.48
Gender distribution (M/F ratio)	1/1.74
Mean weight [kg]	74.53
Normal BMI (*n*)	33
BMI>25 kg/m^2 (*n*)	19

HCV: Hepatitis C virus; *n*: No. of cases; M: Male; F: Female; BMI: Body mass index.

Among the 52 patients in our cohort with confirmed hepatitis C (serology and liver biopsy before the start of the therapy), hepatic steatosis was present in 35 (67.31%) cases; in 17 (32.69%) patients, histopathological examination revealed the presence of hepatic steatosis lesions. Regarding the degree of liver injury, 18 (51.43%) patients had mild steatosis, 11 (31.43%) patients experienced a moderate form (Figure 1) and six (17.14%) patients experienced severe steatosis (Figure 2). All patients with BMI above 25 kg/m^2 had moderate and severe steatosis, which makes us believe that nutrition and weight gain are important factors that contribute to hepatic steatosis.

When evaluating the histopathological appearance of steatosis, 29 (82.86%) patients had predominantly macrovesicular steatosis and only six (17.14%) patients had the microvesicular form.

Steatosis presented an area related distribution with increased presence in the acinar zone 1 and 3 (28 cases) and panacinar in seven cases.

Biochemical investigations have shown that the average values of viremia, ALT and AST were higher in patients with chronic hepatitis C and steatosis than in patients with chronic hepatitis C with steatosis (Table 2), which means that steatosis may be an aggravating factor for chronic lesions caused by the infection with hepatitis C.

Table 2 – ***Mean values of the main biochemical parameters in patients with and without steatosis***

Biochemical parameters (mean values)	With steatosis	Without steatosis
Viremia [IU/mL]	1 258 987	1 058 964
AST [IU/L]	132.99	112
ALT [IU/L]	99.80	87.70

AST: Aspartate transaminase; ALT: Alanine transaminase.

Histopathological examination aimed to correlate the hepatic steatosis with the necroinflammatory activity, respectively to the degree of hepatocyte damage and presence of inflammatory infiltrate within the portobiliary spaces.

The 18 cases of mild steatosis were associated with mild necroinflammatory activity in nine (50%) patients (Figure 3), with moderate activity in seven (38.89%) patients and in two (11.11%) patients with severe activity (Figure 4).

The cases of moderate steatosis (11 patients) were associated with mild necroinflammatory activity in five (45.45%) patients with moderate necroinflammatory activity in four (36.37%) cases and severe activity in two (18.18%) patients. The six cases with severe steatosis were associated with light necroinflammatory activity in two (33.33%) cases, moderate necroinflammatory activity in three (50%) cases and one (16.67%) case with severe activity (Figure 5). The six cases with severe steatosis were associated with light necroinflammatory activity in two (33.33%) cases, moderate necroinflammatory activity in three (50%) cases and one (16.67%) case with severe activity (Figure 5).

The necroinflammatory activity was highly variable from one patient to another and even one portal area to another in the same patient from. Distribution of inflammatory cells varied from case to case, from a barely perceptible inflammatory infiltrate, to large inflammatory infiltrates, sometimes organized in lymphoid nodules. The majority of the cell population of lymphocytes was found within portal spaces; we also highlighted macrophages and even plasmocytes in smaller amounts.

The number of areas affected by the inflammatory process varied from a few up to their full involvement, on the same biopsy. Portal space sizes were both normal and enlarged due to the presence of inflammatory cells infiltrate. In assessing the scale of portal spaces, one must

take into account that in addition to the physiological variability, they can appear smaller and contain lower cell counts because the histological section did not intersect the largest diameter, involving only a small peripheral portion of these spaces. Therefore, in assessing the scale and intensity of the inflammatory infiltrate in these portal spaces, we considered the largest spaces.

Appreciating the association between the steatosis grade and the intensity of the hepatic fibrosis process was done only on GS trichrome staining, which best highlights collagen fibers. Thus, the 18 cases with low steatosis were associated with low fibrosis in 10 (55.56%) patients (Figure 6), with moderate fibrosis in seven (38.89%) patients and severe fibrosis only one (5.55%) patient.

Of the 11 cases of mild steatosis, four (36.36%) patients had mild fibrosis, three (27.28%) patients had moderate fibrosis and four (36.36%) patients had severe fibrosis (Figure 7). The six cases with severe steatosis have been associated with moderate fibrosis in four (66.67%) patients (Figure 8) and with severe fibrosis in two (33.33%) patients.

The reduced fibrosis stage (F1) was characterized by an increasing amount of collagen fibers in the portal spaces, without the presence of fiber expansion to form septa. The stage of moderate fibrosis (F2) was additionally characterized by the presence of fibrous septa started within the portal area and the stage of severe fibrosis was characterized by the presence of thick fiber septa, arranged in bridges on the porto-central trajectory (towards the centrolobular vein), or porto-portal.

The immunohistochemical study evaluated the distribution of inflammatory cells (T-lymphocytes, B-lymphocytes and macrophages/Kupffer cells) depending on the severity of steatosis.

T-lymphocytes have emerged, as we expected, in large numbers in the portal spaces (Figure 9). However, many T-lymphocytes have been identified intralobullarly, regardless of the degree of steatosis (Figure 10). We believe that the presence of intralobular T-lymphocytes is not due to steatosis but to immunological factor stimulated by lesions induced by hepatitis C.

B-lymphocytes occurred generally in smaller numbers than T-lymphocytes, disseminated mainly in the port area and the first area of the liver lobule (Figure 11). Lymphoid nodules were identified in the portal area in some cases, formed mainly of B-lymphocytes.

Portal spaces macrophages appeared in much smaller numbers than T-lymphocytes (Figure 12), regardless of the degree of steatosis. Their number was increased in those cases where necrotic activity was intense. Kupffer cells appeared much hypertrophied in most cases of hepatitis C. The number and size of Kupffer cells appeared much reduced within areas steatosis compared to other areas of hepatic parenchyma (Figure 13).

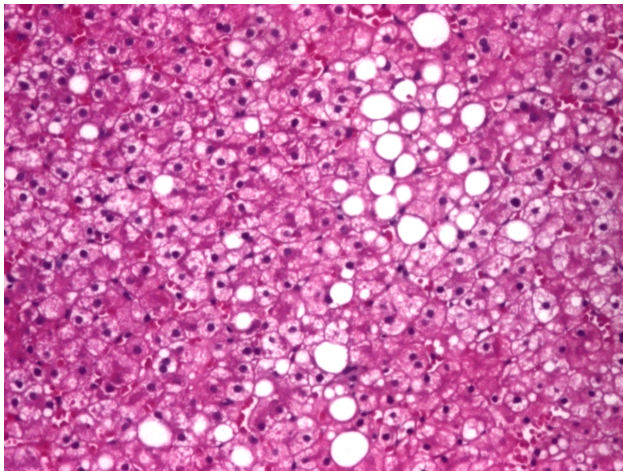

Figure 1 – ***Microscopic image of moderate steatosis. HE staining, ×200.***

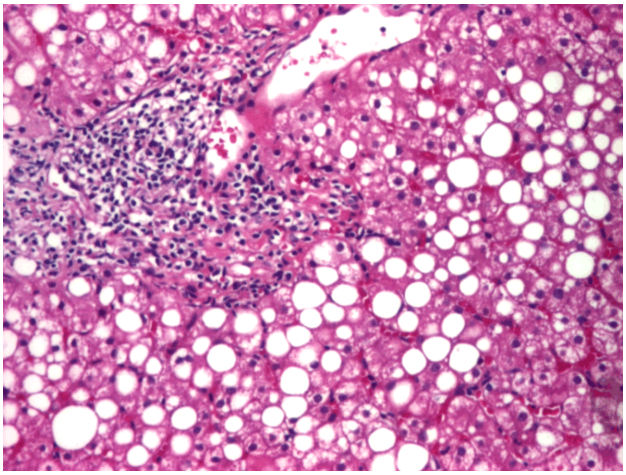

Figure 2 – ***Severe steatosis. HE staining, ×200.***

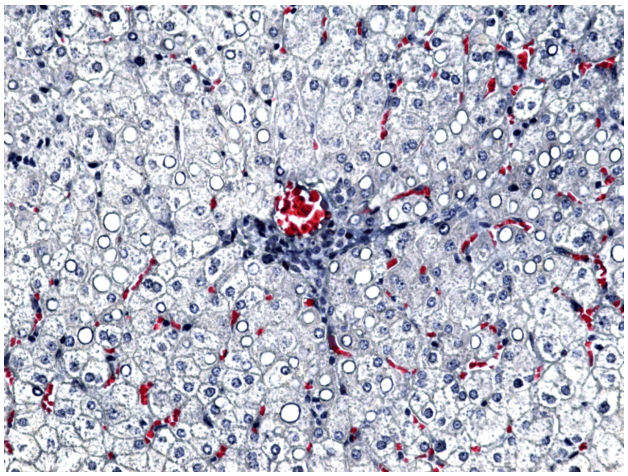

Figure 3 – ***Low steatosis with light necroinflammatory activity. GS trichrome staining, ×200.***

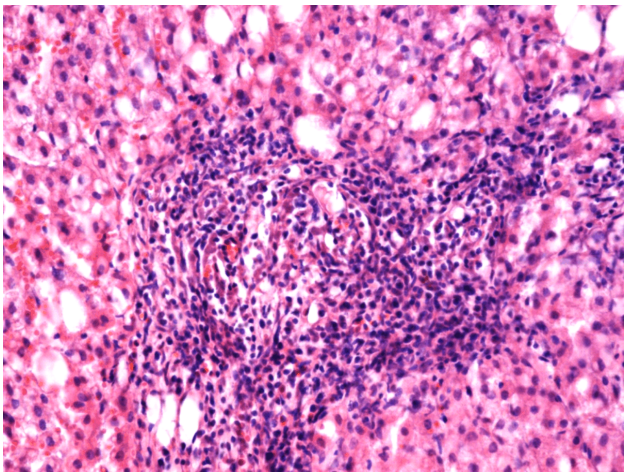

Figure 4 – ***Low steatosis with intense necroinflammatory activity. HE staining, ×100.***

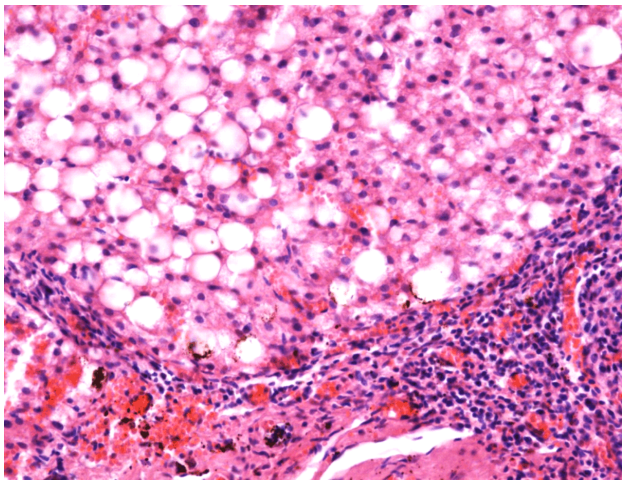

Figure 5 – ***Severe steatosis associated with intense necro-inflammatory activity. HE staining, ×200.***

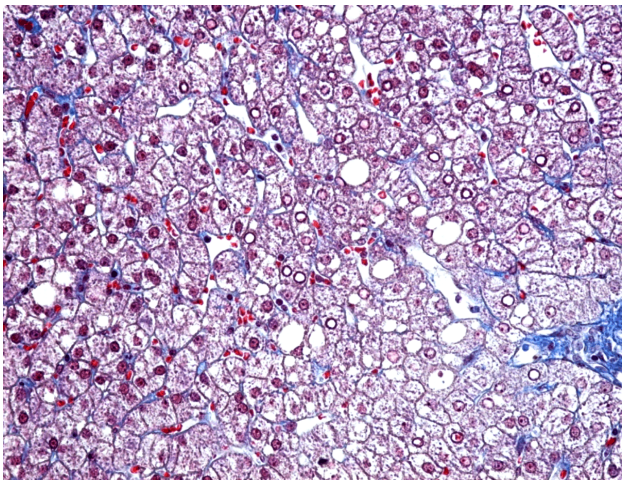

Figure 6 – ***Microscopic image of chronic viral C hepatitis and low fibrosis and steatosis. GS trichrome staining, ×200.***

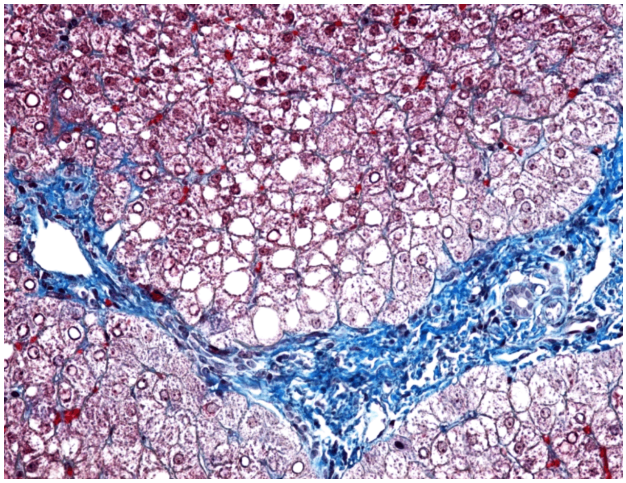

Figure 7 – ***Image of moderate steatosis associated with severe fibrosis. GS trichrome staining, ×200.***

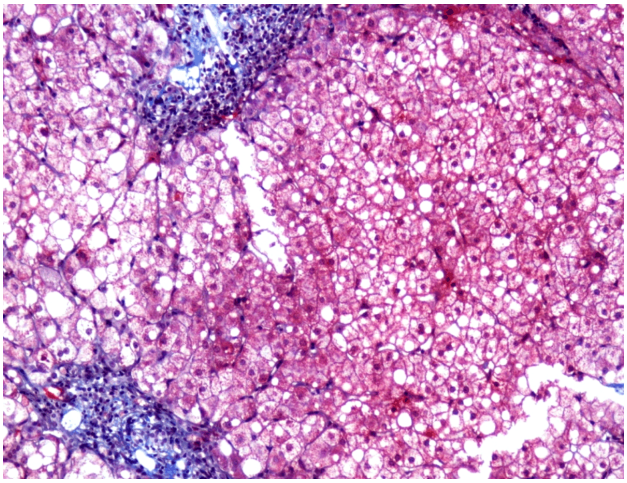

Figure 8 – ***Severe steatosis, mainly microvesicular, associated with moderate fibrosis. GS trichrome staining, ×100.***

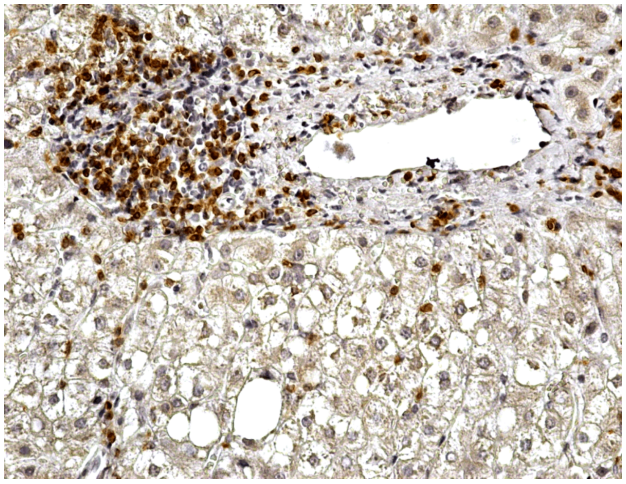

Figure 9 – ***Low steatosis associated with an abundant inflammatory infiltrate in the portal area, consisting of a large number of T-lymphocytes. Immunostaining with anti-CD3 antibody, ×200.***

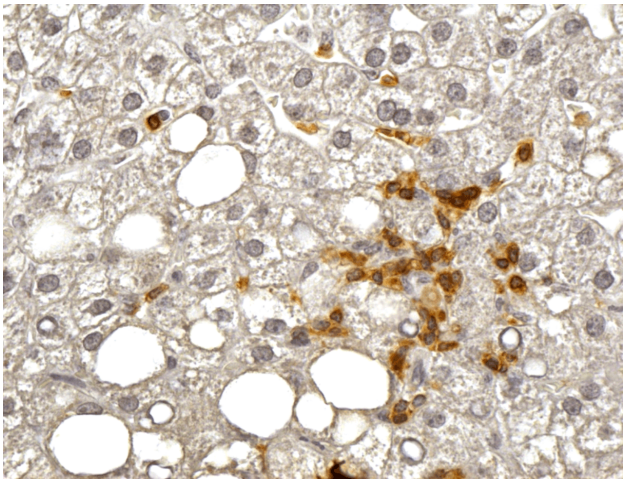

Figure 10 – ***Macro- and microvesicular moderate steatosis associated with numerous T-lymphocytes present among cords of intralobular Remack hepatocyte. Immunostaining with anti-CD3 antibody, ×400.***

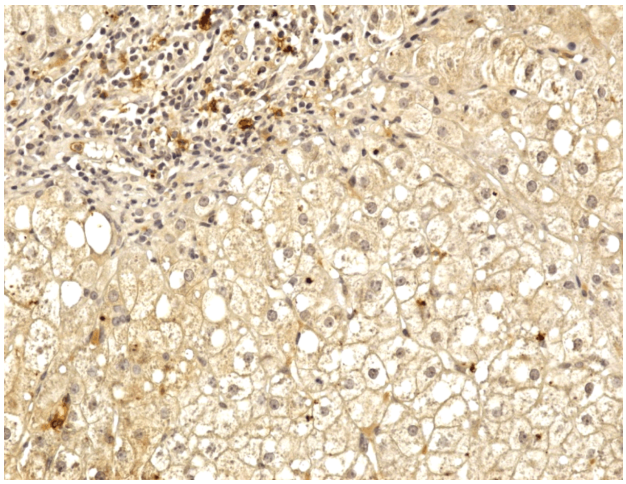

Figure 11 – ***Chronic hepatitis C with moderate necro-inflammatory activity, with numerous diffusely disseminated B-lymphocytes within the periportal portal space, associated with macro- and micronodular moderate steatosis. Immunostaining with anti-CD20 antibody, ×200.***

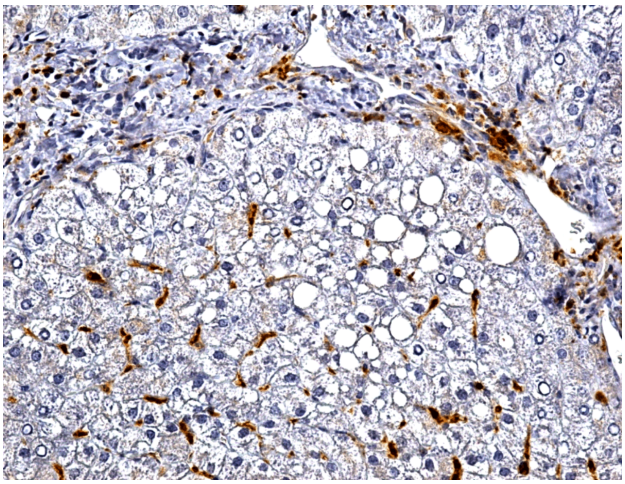

Figure 12 – ***Severe chronic hepatitis with thick porto-portal fibrous bands and intense necroinflammatory activity associated with light macrovesicular steatosis injuries. The cells of the monocyte–macrophage system are present in both portal spaces and intralobularly (Kupffer cells). Immunostaining with anti-CD68 antibody, ×200.***

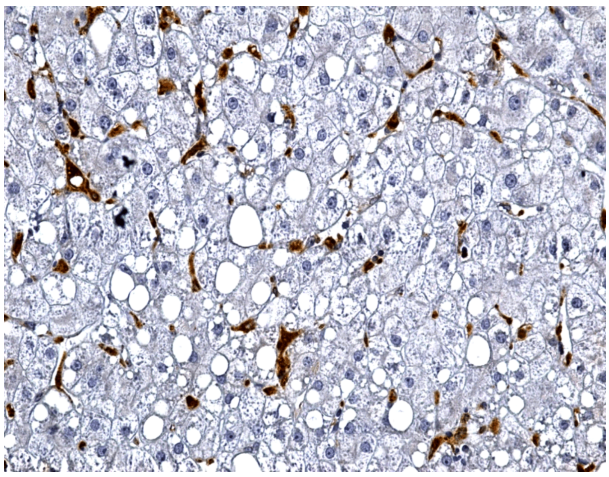

Figure 13 – ***Image of medium steatosis, predominantly macrovesicular, present in the second area of the hepatic lobule, associated with a reduction in the number of Kupffer cells. Immunostaining with anti-CD20 antibody, ×200.***

Discussion

The aim of this paper was identifying factors correlated with the presence of liver steatosis. We evaluated the presence of steatosis in patients with chronic viral C hepatitis, the association between steatosis and viral aspects, and relationship between steatosis and fibrosis.

Several studies have reported that hepatic steatosis is a microscopic lesion frequently identified in patients with chronic hepatitis C. According to some authors [16, 17], the prevalence of steatosis varies between 40% and 86% in patients infected with hepatitis C. In our study, cases of chronic hepatitis C associated with steatosis represented 67.31%. The presence of lesions of steatosis in all patients with a BMI above 25 kg/m^2 makes us believe that obesity and metabolic disturbances are some of the factors underlying the etiopathogenic occurrence of hepatic steatosis. However, the presence of lesions of steatosis in patients with normal weight, infected with hepatitis, shows that this infection is another factor. Our observations are supported by other studies showing that three mechanisms for steatosis exist in patients infected with HCV [5, 16]:

- a metabolic mechanism;
- a viral mechanism directly related to the cytopathogenic effect of the hepatitis C virus;
- a combined mechanism that appears to be the most frequent and in which both viral and the metabolic mechanism are involved in varying proportions.

Much of the life cycle of HCV interferes with lipid and carbohydrate metabolism [6]. Some viral proteins such as the "core" protein and the non-structural protein 5A (NS-5A) interfere directly with the lipid and carbohydrate metabolism of the infected hepatocytes [18–21].

For the identification of the intimate mechanism by which the "core" protein of HCV interacts with hepatocytes in the genesis of steatosis, numerous studies were performed on cultured cells and transgenic mice. These experiments demonstrated that intracellular lipid accumulation is even more intense as the "core" protein is higher expressed [22]. The "core" protein interacts with apolipoproteins A1 and A2, disturbing the lipid metabolism of hepatocytes, resulting in intracellular accumulation of triglycerides [8]. Also, HCV "core" protein interacts with several nuclear receptors that regulate many cellular genes involved in lipid synthesis, thus promoting *de novo* lipogenesis [23, 24].

Other studies have shown that the "core" protein inhibits the activity of microsomal enzymes involved in the formation and transfer of triglycerides and very low-density lipoprotein, which causes them to be stored in the cytoplasm, thus facilitating steatosis [25]. The "core" protein enters the mitochondria, disturbing its activity, with an increased production of reactive oxygen species [26, 27] and the accumulation of fatty acids in the mitochondria [28].

Interrelations between HCV infection, steatosis and

the metabolic syndrome are quite complex and incompletely known. According to some studies, the severity of steatosis correlates with the level of viral replication [4]. Other studies argue that the appearance of lesions of steatosis and intensity depend on the HCV genotype. Infection with HCV genotypes 2 and 3 lead to steatosis in the absence of metabolic syndrome [29, 30], whereas the infection with HCV genotype 1 leads to steatosis only in the presence of metabolic syndrome (obesity, hyperlipidemia) [31].

Unfortunately, in our study, we were able to detect viral genotype but the majority of patients with liver steatosis presented a higher viremia, a BMI over 25 kg/m^2 and high values of AST and ALT. Some studies showed that steatosis was not associated with BMI [32]; other epidemiological studies have shown similar data, showing that this obesity was associated with hepatic steatosis and elevated levels of ALT [33–35].

Regarding microscopic lesions, we noted a variety of lesions, the intensity of steatosis associating only a small number of cases necroinflammatory activity and hepatic fibrilogenesis process. We identified serious chronic hepatitis C, with a very high HAI score, without injury steatosis, as we have identified moderate forms of chronic hepatitis C associated with intense steatosis. However, we believe that hepatic steatosis associated with chronic hepatitis C virus worsens hepatic dysfunction and increases the risk of progression of the disease to severe forms of hepatitis or cirrhosis. By some estimations, the association of chronic hepatitis C with steatosis aggravates the risk of faster progress of the disease and causes a poor response to treatment with interferon alpha [36, 37].

Immunohistochemical study we conducted allowed us to highlight T- and B-lymphocytes and macrophages within the inflammatory infiltrate of the main portal spaces. We appreciated their participation to the necroinflammatory activity. Besides porto-biliary spaces, we identified T-cells in the liver parenchyma, even in areas with steatosis; we also noticed a reduction in the number and size of Kupffer cells in areas of steatosis. We believe that the involvement of immune cells in development of liver steatosis is less investigated.

Several studies have shown that obesity associated with chronic hepatitis determines an abnormality of cytokines and activates several signaling pathways, which secondary induce a variety of biochemical processes that result in an increase in some inflammatory biomarkers (C-reactive protein, interleukin-6, tumor necrosis factor-alpha), including increased oxidative stress [38, 39]. Like other authors [40, 41], we believe that between chronic hepatitis, obesity and steatosis there are complex interrelations, some still insufficiently known.

Conclusions

In our study, 52 patients with a clinical, serological and histopathological diagnosis of chronic hepatitis C, 35 (67.31%) had hepatic steatosis. Of these, 18 (51.43%) patients had mild steatosis, 11 (31.43%) patients had moderate steatosis and six (17.14%) patients experienced severe steatosis. All patients with a BMI greater than 25 kg/m^2 had moderate and severe steatosis. After histopathological appearance of steatosis, 29 (82.86%) patients had steatosis, predominantly macrovesicular, and only six (17.14%) patients had a microvesicular form. Steatosis intensity did not correlate with the severity of liver damage induced by chronic infection with HCV.

Conflict of interests

The authors declare that they have no conflict of interests.

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