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Hepatic injuries resulting from chronic alcohol abuse identified by forensics

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Abstract

Alcohol intake is largely spread all over the world, although it is well-known that it causes important changes of the liver, from liver steatosis, hepatitis to liver cirrhosis. The study we performed on 93 patients deceased in suspicious circumstances and brought to the Institute of Forensic Medicine, Iaşi, Romania, confirmed through the determination of Ethyl glucuronide, that these were alcohol consumers. The macroscopic analysis during necropsy highlighted the presence of alcoholic liver disease (ALD), while microscopy studies confirmed the macroscopic observations. The immunohistochemical studies showed the existence of a chronic liver inflammation, the transdifferentiation of stellate hepatic cells, the activation of Kupffer cells, important changes of the hepatic vascular network, hepatocyte necrosis, lipid loadings and the progression of liver fibrosis process.

Keywords: chronic alcohol consumption, liver changes, Ethyl glucuronide, blood, histopathology.

Introduction

Alcohol abuse, through the medical consequences it generates, is an important public health issue [1–3]. The study of existing protocols in identifying the markers of chronic alcohol consumption performed in laboratories in Romania and other countries showed that alcohol directly affects the most important organs and tissues, leading to systemic dysfunctions, which can have severe health consequences, from a declining quality of life to death [4–7]. Chronic alcohol users may experience significant functional and morphological changes [8–11]. On a long term, all tissues and organs are vulnerable to alcohol abuse, but almost all medical consequences are only partially reversible with abstinence [12–15].

Fatty liver or hepatic steatosis is the initial stage of alcoholic liver disease (ALD), being the most common liver disease associated with alcohol abuse. It is characterized by excessive accumulation of fat inside the liver cells, which makes it difficult for the liver to function. Steatosis usually has no symptoms, although the liver may be enlarged, which causes discomfort in the right abdominal flank. Fatty liver occurs quite early in most people with alcohol abuse but can go into remission after abstinence [2].

Alcoholic hepatitis is an inflammation of the liver accompanied by damage of the liver cells. Up to 35% of heavy alcohol users develop chronic, moderate, or severe hepatitis. The damage is partially reversible over time if you give up alcohol. In the severe, acute form, the disease can set in suddenly, after the ingestion of a large amount of alcohol in a short time and can cause immediate complications leading to death.

Alcoholic cirrhosis is the most severe complication of ALD. In cirrhosis, normal liver cells are replaced by dysfunctional fibrocystic cells. Between 10% and 20% of heavy drinkers develop cirrhosis, usually after 10 or more years of drinking. Any toxic that attacks the liver over several years can cause it to form scar tissue. Fibrosis is the first stage of scar tissue formation, but when fibrosis accumulates and occupies the major organ, cirrhosis sets in [16–21].

Aim

The present study has aimed to study the forensic cases of the Institute of Forensic Medicine, Iaşi, Romania, in real time and select cases with a history of chronic alcohol consumption: (*i*) select the deceased cases, alleged victims of chronic Ethanol consumption; (*ii*) quantify Ethyl glucuronide (EtG), a biomarker that is specific to chronic alcohol consumption, in blood samples collected from the corpse, using gas-chromatographic method; (*iii*) correlate the microscopic and immunohistochemical (IHC) lesions with the level of EtG detected in blood samples using the gas-chromatographic method.

Materials and Methods

The study was performed on 93 corpses, within the Institute of Forensic Medicine (IFM) Iaşi morgue, between June 15, 2020, and December 12, 2020, according to the Approval No. 9421/12.06.2020 issued by the Research

Ethics Committee within Grigore T. Popa University of Medicine and Pharmacy, Iaşi.

The survey data showed that the patients included in the study were chronic consumers of alcohol.

The following hepatic lesions were macroscopically found during the necropsy examination: hepatic steatosis, alcoholic hepatitis, and cirrhosis.

The final cause of death of those mentioned was acute cardio-respiratory failure, which appeared because of hepatic lesions detected during the necropsy. The hepatic lesions were dominant in the cause of death.

All forensic necropsies were performed according to the Iaşi County Police Inspectorate (IPJ Iaşi) order documents.

The data (focusing on age, demographical features, alcohol consumption and other diseases) were centralized in Statistical Package for the Social Sciences (SPSS) 18.0 database and processed with the statistical functions for which they are suitable, at the significance threshold of 95%.

The blood samples were harvested from the studied cases at a maximum of four hours after death, for the determination of the level of EtG by gas-chromatographic method.

During necropsy, there were harvested liver fragments of approximately 2/1/2 cm from every corpse, for the microscopic examination and the clear determination of the death cause. The harvested liver fragments were immediately put into a fixing solution of 10% formalin, with a neutral pH, for 48–72 hours and then included in paraffin, according to the histopathological protocol. Then, there were performed microtome sections of 4 µm thickness stained with Hematoxylin–Eosin (HE) and with Goldner–Szekely (GS) trichrome. For the IHC study, we used the following antibodies: anti-cluster of differentiation (CD)3 (monoclonal mouse anti-human CD3, clone F7.2.38, 1/25 dilution, Dako), anti-CD20 (monoclonal mouse anti-human CD20cy, clone L26, 1/50 dilution, Dako), anti-CD68 (monoclonal anti-human CD68, clone KP1, 1/100 dilution, Dako), anti-CD31 (monoclonal mouse anti-human CD31, endothelial cell, clone JC70A, 1/50 dilution, Dako), anti-CD34 (monoclonal mouse anti-human CD34 Class II, clone QBEnd 10, 1/50 dilution, Dako) and anti-alpha-smooth muscle actin (α-SMA) (monoclonal mouse anti-human SMA, clone 1A4, 1/100 dilution, Dako). By the IHC study, we proposed to investigate the inflammatory reaction in the liver, including the reaction of Kupffer cells, the reaction of myofibroblast and Ito cells, as well as the changes of the intra- and extra-lobular blood vessel network in patients with ALD.

The analysis of variance (ANOVA) test was used to evaluate descriptive statistical indicators: minimum, maximum, average, median, standard deviation (SD), standard error of the mean (SEM), variance. The skewness test ($-2 < p < 2$) validates the normality of the value series. In calculating the significant difference between two means, the Student's *t*-test considers the measurement of variability and the weight of observations for series of values with normal distributions. *Chi*-squared (χ^2) is a nonparametric test comparing two or more frequency distributions from the same population, applied when expected events are excluded.

Results

Starting from the hypothesis that alcohol is a widely accepted drug by the society and consumed by individuals of various ages (starting with children and adolescents up to the elderly), in the present study we proposed to analyze the age of the individuals deceased in unclear conditions, with the diagnosis of ALD, as a result of alcohol abuse, in whom there was required a forensic expertise for a clear determination of the death cause, of associated lesions and the symptoms leading to death.

The age distribution in our group allowed us to observe that death caused by alcohol abuse may occur in individuals aged from 18 to 89 years old. If under the age of 50 years old, deaths caused by ALD were more rare (1–9 cases per age decade), after the age of 50 years old, deaths significantly increased, thus showing that the alcohol abuse leads to progressive liver lesions, with a cumulative effect. After the age of 50 years old, there were recorded 76 cases, representing 81.72% of the entire group of patients (Figure 1).

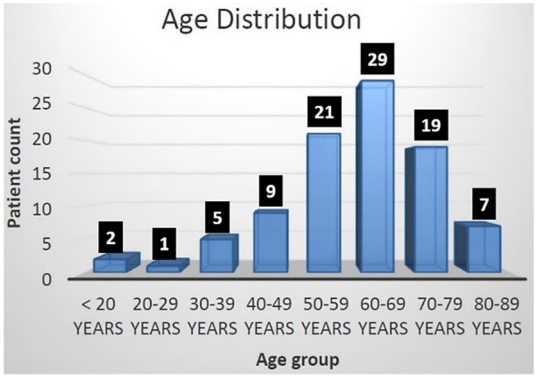

Figure 1 – ***Distribution of patients according to age.***

Regarding the sex of patients with ALD, in our study we observed that a number of 70 individuals, representing almost 75%, were males and only 23 (about 25%) were females (Figure 2). By studying the distribution of the group of patients according to their social environment, we observed that most patients with ALD, namely 61 (66%), were from the rural area and 32 (34%) from the urban area (Figure 3).

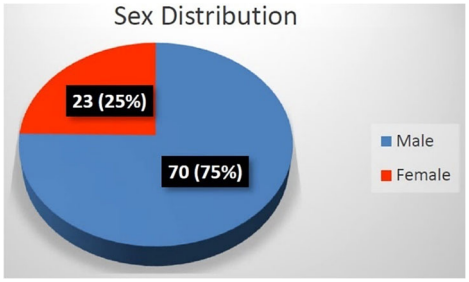

Figure 2 – ***Distribution of deceased patients with ALD according to sex (n; %). ALD: Alcoholic liver disease.***

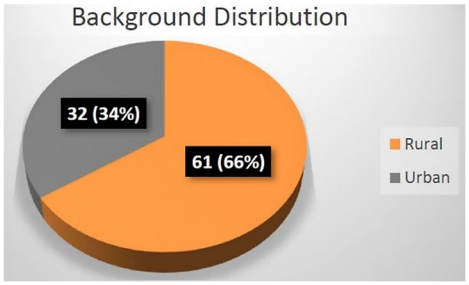

Figure 3 – ***Distribution of patients with ALD according to the environment (n; %). ALD: Alcoholic liver disease.***

For highlighting the excessive alcohol intake by the patients in our study group, we identified in the blood, through gas-chromatography, the presence of a stable alcohol compound, namely EtG. EtG is a very stable compound resulting from the alcohol metabolization, being traceable in the liquid environments for a long period after complete alcohol elimination from the body.

After the macroscopic aspect of the liver, we identified in the deceased patients with ALD in our group the presence of three distinct sections: hepatosteatosis, alcoholic hepatitis and cirrhosis. The determination of EtG level in the blood of corpses included in the study and the correlation with the type of liver disease, showed no significant differences between the three types of liver diseases.

However, we have noticed a mean level of EtG significantly higher than the EtG–D5 internal standard (500 ng/mL) in subjects with alcoholic hepatitis (738± 379 ng/mL; p=0.028), hepatic steatosis (709±210 ng/mL; p=0.043), cirrhosis (735±371 ng/mL; p=0.049) (Table 1; Figure 4). (500 ng/mL EtG–D5 is the internal standard provided by the gas-chromatograph with mass spectrometry).

Table 1 – ***Average level of EtG in the blood depending on hepatic complications***

Liver diseases	*n* (%)	Concentration of EtG [ng/mL]	Mean / min.–max. [ng/mL]	Student's *t*-test *p*
Hepatic steatosis	46 (49.5%)	709±210	1264 / 9–5932	*0.043*
Alcoholic hepatitis	32 (34.4%)	738±379	1834 / 9–7384	*0.028*
Cirrhosis	15 (16.1%)	735±371	1876 / 9–7384	*0.049*

EtG: Ethyl glucuronide; *n*: No. of cases.

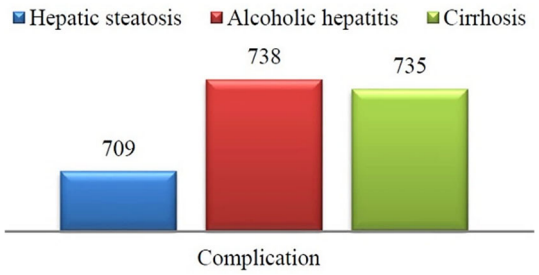

Figure 4 – ***Mean levels of EtG [ng/mL] in blood samples depending on the associated hepatic pathology. EtG: Ethyl glucuronide.***

The histopathological and IHC examinations of the liver confirmed the macroscopic diagnosis of liver lesions described during necropsy. We should mention that the microscopic lesions identified by us were much more complex than they seemed in the macroscopic examination, in the same patient being identified both lipid accumulation in the hepatocytes (steatosis), and fibrotic lesions or accumulations of inflammatory cells in the portobiliary space.

The most frequent liver lesions were steatosis ones, characterized by the accumulation in higher or lower quantities of lipids in the hepatocytes. Of the 93 cases of ALD, we identified steatosis as the main lesion in 46 deceased individuals, representing about 49.5%; steatosis changes of low intensity were also identified in some cases of chronic hepatitis or cirrhosis. Most often, we identified forms of macrovacuolar steatosis, but in about 25% of the steatosis cases, this was of the microvacuolar type (Figures 5 and 6). Some cases of liver steatosis were associated with lesions of granular liver degenerescence or even with isolated hepatocyte necrosis, microscopic aspects showing a more advanced stage of the liver disease.

Alcoholic hepatitis was identified in 32 cases, characterized by granular and vacuolar degenerescence of hepatocytes, limited hepatocyte necrosis, the presence of an inflammatory infiltrate made of neutrophil granulocytes, lymphocytes, and macrophages, mainly localized in the Kiernan space, moderate perivenular and pericellular fibrosis, moderate steatosis (Figures 7 and 8).

Liver cirrhosis was identified in 15 individuals and was characterized by the development of an intense process of collagen fibrosis, mainly in the portobilliary spaces, with the presence of porto–portal and intralobular bridging fibrosis, up to the central lobular vein. The development of the fibrous process led to the change of the liver parenchyma, with the occurrence of parenchymatous nodular, non-functional, limited structures of fibrous conjunctive tissue, where hepatocytes presented phenomena of necrosis, vacuolar degenerescence or lipid charge (Figures 9 and 10).

The IHC examinations highlighted the presence of liver inflammatory infiltrates in all cases of ALD, regardless of the disease type (steatosis, hepatitis or cirrhosis). In the case of liver steatosis, the inflammatory infiltrates with T- and B-lymphocytes were low; of the two types of lymphocytes, the most numerous were T-lymphocytes, identified both in the extralobular spaces (Kiernan) and in the intralobular ones (Figures 11 and 12).

In the cases of alcoholic hepatitis, the reaction of T-lymphocytes was more intense, while the reaction of B-lymphocytes remained low (Figures 13 and 14).

The cells of the macrophage system, represented by Kupffer cells and the macrophages in the Kiernan space had a different reaction in the deceased individuals with ALD. In our study, the Kupffer cells appeared hypertrophied, with a large nucleus, sometimes hypochromic, with a heterogenous abundant cytoplasm, with a vacuolar aspect, intensely positive to anti-CD68, thus showing an important increase of the lysosomes (Figure 15). Instead, the macrophages in the Kiernan space appeared in small dimensions, with a poorer reaction to anti-CD68 antibody, proof of a lower phagocyte activity, in comparison to the Kupffer cells (Figure 16).

The reaction of stellate Ito cells in patients with ALD was analyzed by using the anti-α-SMA antibody. Also called liver stellate cells, these cells are physiologically arranged between the endothelial cells of sinusoidal liver capillaries and hepatocytes (Disse space). These cells have a small property, namely to deposit vitamin A under the form of cytoplasm drops. Recent studies showed that, under certain pathological conditions, liver stellate cells transdifferentiate into proliferative, migratory, and contractile myofibroblasts, secreting various molecules specific to the extracellular conjunctive matrix, especially procollagen proteins that accumulate in the liver as a scar tissue. In our study, we highlighted processes of transdifferentiation of stellate cells even from the initial stage of ALD (liver steatosis) (Figure 17). Also, in alcoholic liver cirrhosis, we observed a high number of myofibroblasts in the Kiernan spaces (Figure 18), some of these being possible liver stellate cells migrated in this area.

Using some IHC markers for the endothelial cells allowed us to highlight the change of the hepatic micro-circulation in ALD. In our study, we observed a significant

reduction of the intralobular sinusoidal capillaries, even from the stage of steatosis of the ALD and the development of new blood vessels in the portal spaces or in the porto–portal conjunctive septa (Figures 19 and 20).

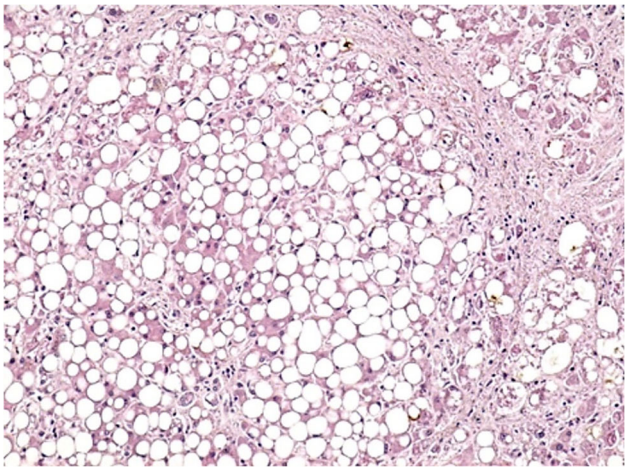

Figure 5 – ***Image of macrovascular steatosis associated with perilobular fibrosis. HE staining, ×100. HE: Hematoxylin–Eosin.***

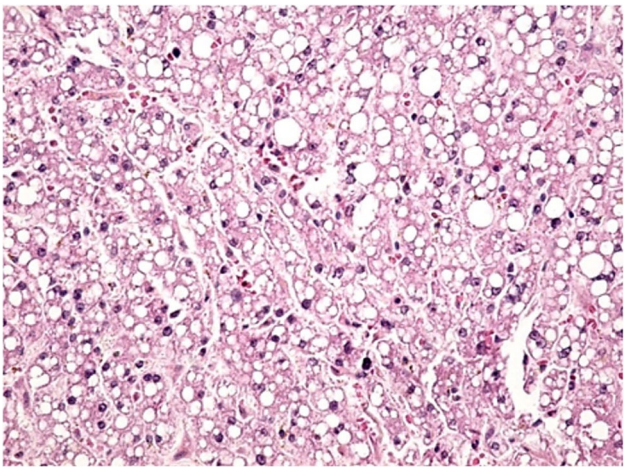

Figure 6 – ***Image of liver with macro- and microvacuolar steatosis. HE staining, ×100.***

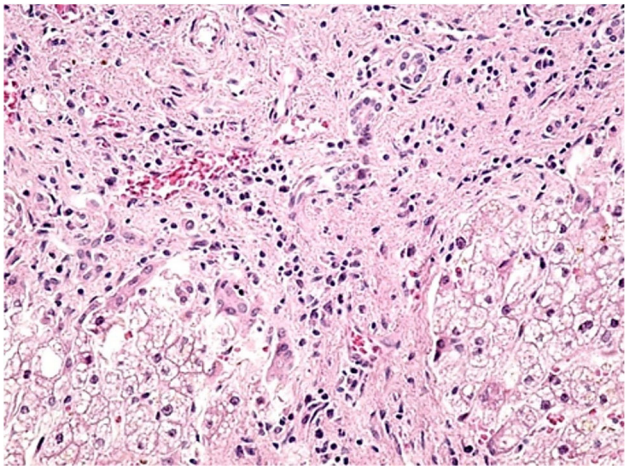

Figure 7 – ***Image of alcoholic hepatitis characterized by vacuolar degenerescence of hepatocytes, moderated hepatocyte necrosis and the presence of a moderate inflammatory infiltrate in the Kiernan space, associated with a deposit of collagen fibers. HE staining, ×100.***

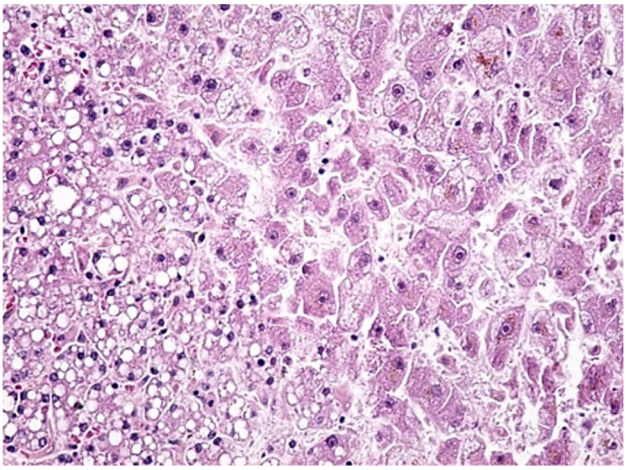

Figure 8 – ***Liver parenchyma in a case of alcoholic hepatitis, where we may observe the ballooning of hepatocytes, vacuolar degenerescence, associated with a microvascular steatosis. HE staining, ×200.***

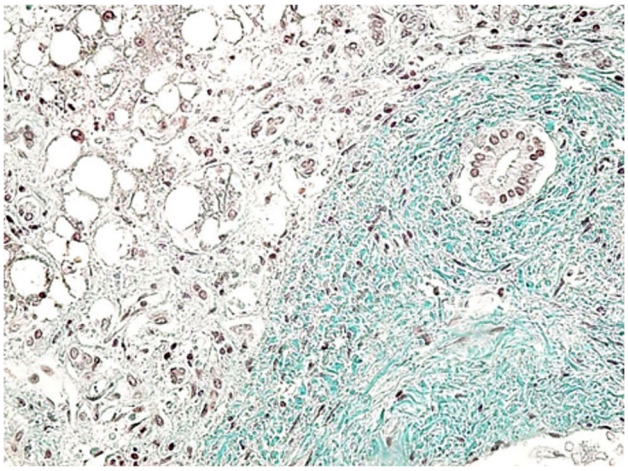

Figure 9 – ***Image of liver cirrhosis, characterized by the abundant development of the fibrous conjunctive tissue in the Kiernan spaces, and also in the intralobular spaces, associated with hepatocyte necrosis and macrovacuolar steatosis. GS trichrome staining, ×200. GS: Goldner–Szekely.***

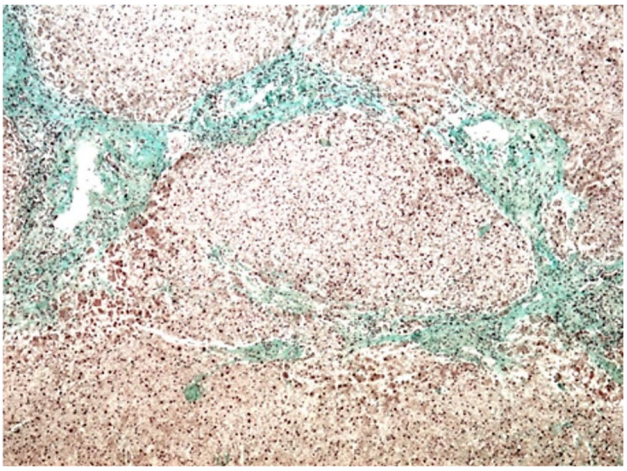

Figure 10 – ***Liver cirrhosis: regeneration nodule. GS trichrome staining, ×40.***

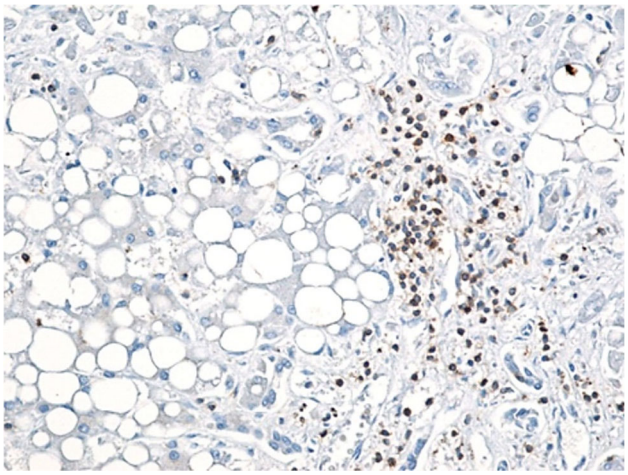

Figure 11 – ***Area of liver steatosis with a moderate inflammatory infiltrate in the Kiernan space. Anti-CD3 antibody immunomarking, ×200. CD3: Cluster of differentiation 3.***

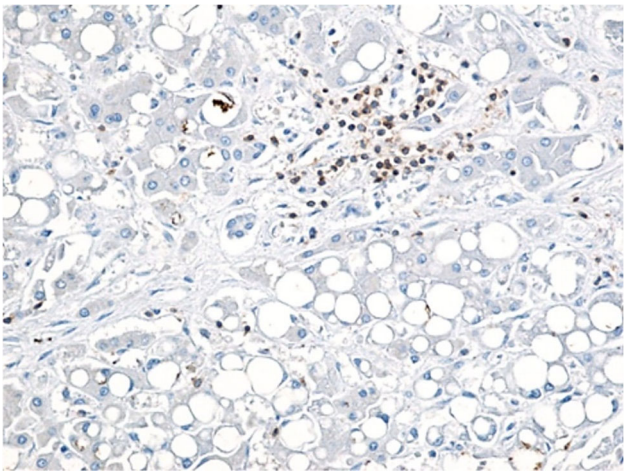

Figure 12 – ***Image of liver steatosis with rare B-lymphocytes in the Kiernan portal space. Anti-CD20 antibody immunomarking, ×200. CD20: Cluster of differentiation 20.***

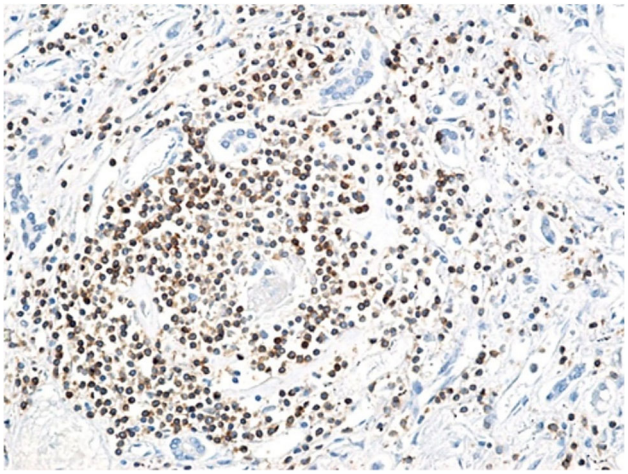

Figure 13 – ***Abundant inflammatory infiltrate in the Kiernan space, mainly formed of T-lymphocytes, in a case of alcoholic hepatitis. Anti-CD3 antibody immunomarking, ×200.***

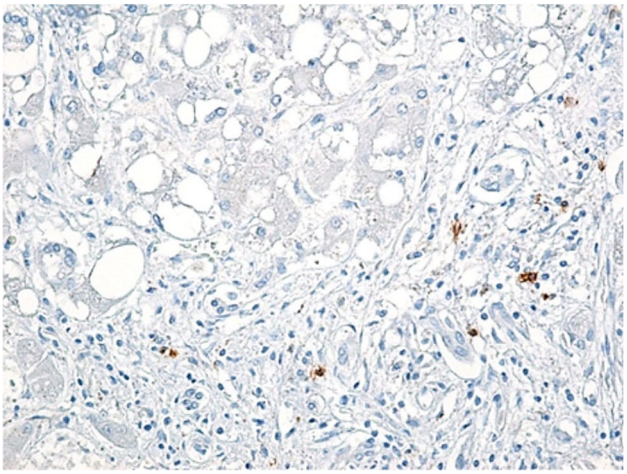

Figure 14 – ***Alcoholic hepatitis with rare B-lymphocytes in the Kiernan space. Anti-CD20 antibody immunomarking, ×200. CD20: Cluster of differentiation 20.***

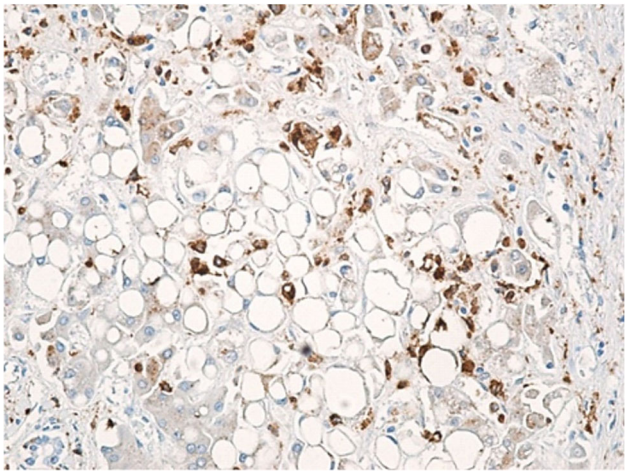

Figure 15 – ***Area of steatohepatitis with numerous hypertrophied Kupffer cells, with vacuolar cytoplasm. Anti-CD68 antibody immunomarking, ×200. CD68: Cluster of differentiation 68.***

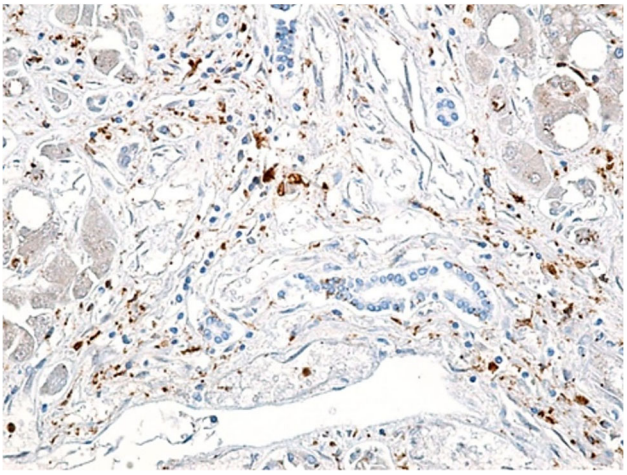

Figure 16 – ***Kiernan space with numerous small-sized macrophages, in a case of alcoholic hepatitis. Anti-CD68 antibody immunomarking, ×200.***

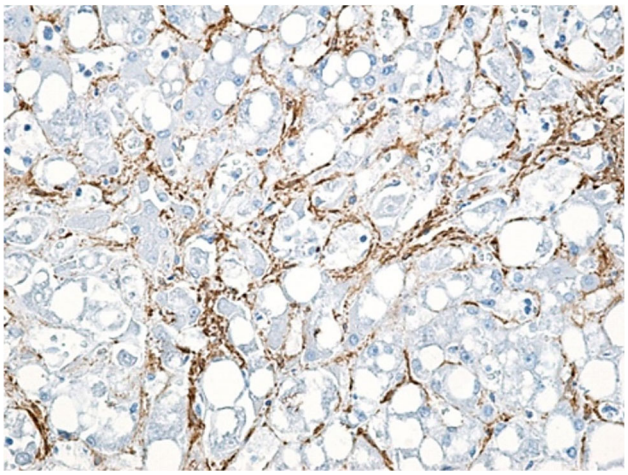

Figure 17 – ***Intense reaction of liver stellate cells to anti-α-SMA antibody in a case of liver steatosis. Anti-α-SMA antibody immunomarking, ×200. α-SMA: Alpha-smooth muscle actin.***

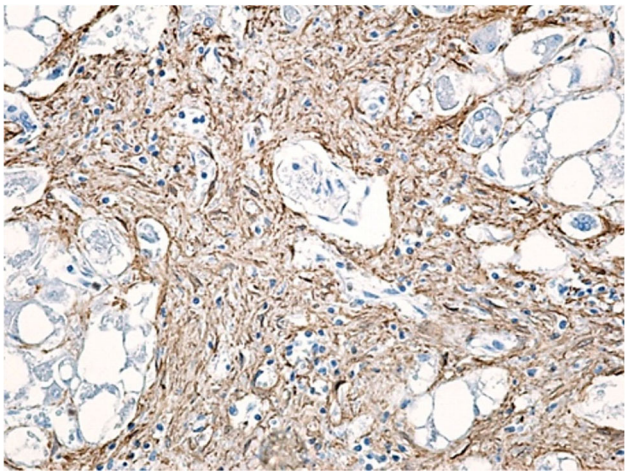

Figure 18 – ***Numerous myofibroblast cells arranged in the Kiernan space, in a case of alcoholic liver cirrhosis. Anti-α-SMA antibody immunomarking, ×200.***

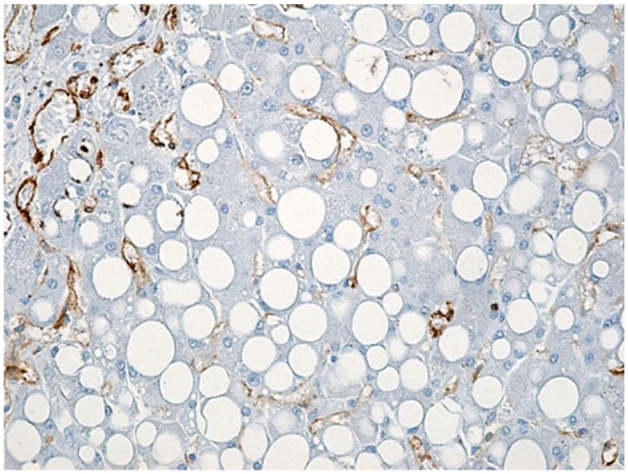

Figure 19 – ***Macrovascular liver steatosis associated with a low number of intralobular sinusoidal capillaries. Anti-CD31 antibody immunomarking, ×200. CD31: Cluster of differentiation 31.***

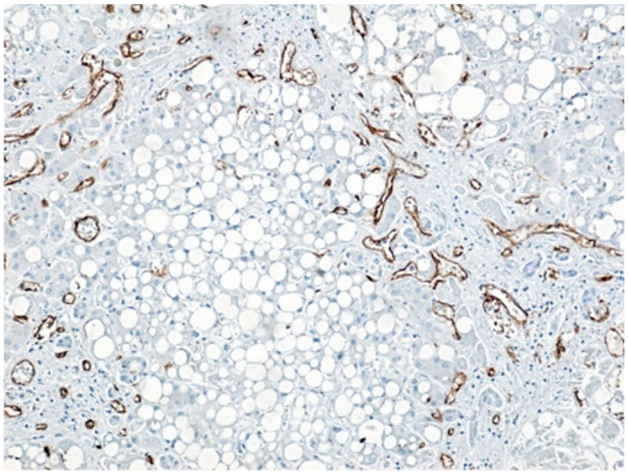

Figure 20 – ***Image of steatohepatitis where we may observe the reduction of the intralobular sinusoidal capillaries and the increase of the number of blood vessels in the Kiernan space and the porto–portal conjunctive septa. Anti-CD34 antibody immunomarking, ×100. CD34: Cluster of differentiation 34.***

Discussions

Alcohol is a major risk factor for lots of chronic diseases and one of the main causes of "predictable" morbidity and mortality all over the world. There was estimated that, worldwide, alcohol is responsible for 2.5–3.3 million deaths every year, namely almost 6% of all global deaths [22–24]. A report of *World Health Organization* (WHO) regarding the relation between alcohol and health showed that alcohol abuse is responsible for at least 60 major types of acute or chronic diseases.

The ALD progression is complex and depends not only on the quantity and rhythm of alcohol intake, but also on a variety of genetic and environmental factors. In general, men who ingest more than 100 g Ethanol every day, for over a 5-year period, present the highest risk in developing alcoholic hepatitis; nevertheless, also women can develop alcoholic hepatitis after ingesting small Ethanol quantities for shorter periods of time [25, 26].

Some studies appreciate that approximately two billion people worldwide have an excessive alcohol intake [27]. Only in the USA, ALD affects more than 10 million Americans every year, the treatment for medical conditions caused by excessive alcohol intake reaching over 166 billion dollars every year [28]. *Centers for Disease Control and Prevention* in the US estimated that deaths caused by alcohol exceeded the deaths caused by chronic diseases [29].

In the last years, the excessive alcohol intake dramatically increased in adolescents and young adults, thus becoming both a health problem and an economic one, as it leads to an increase in the number of work incapacitated days [30, 31].

In our study, we identified two cases of ALD (steatosis) under the age of 20 years old, which makes us consider that these young people started consuming alcohol from childhood.

More studies obtained similar data. In many countries, it was observed that the starting point for alcohol intake decreased under the age of 10 years old; some epidemiological investigations in children and adolescents showed that the alcohol intake started before the age of 15 years old. According to some *WHO* reports, the prevalence of

alcohol intake in young people is higher in Europe and the US [32, 33].

Excessive alcohol intake affects almost all systems and organs; nevertheless, the liver is the most affected organ, as it is the main place of alcohol metabolization. Alcohol abuse may cause a large spectrum of hepatic lesions, collectively called ALDs, including alcohol liver steatosis, alcoholic hepatitis, and cirrhosis [34, 35].

More studies showed that the pathogeny of ALD is complex, and it is triggered by the damage of hepatocytes through a direct action of alcohol and its metabolites on hepatocytes, recruiting and activating inborn immune cells of the liver, activating stellate cells and myofibroblasts in the liver, thus leading to fibrosis and alcoholic cirrhosis [36–39].

In our study, we identified mainly steatosis lesions in 46 individuals, alcoholic hepatitis in 32 cases and cirrhosis in 13 patients.

Steatosis is the earliest and most frequent response of the liver in chronic or excessive alcohol intake. For a long time, liver steatosis was considered a relatively harmless secondary effect of alcohol intake, but recent studies showed that individuals with alcohol induced steatosis are vulnerable in developing alcoholic steatohepatitis, liver fibrosis, cirrhosis and even liver carcinoma [40–43]. Still, liver steatosis is completely reversible if alcohol intake is interrupted completely [38].

Alcoholic hepatitis is a chronic inflammatory disease, associated with high morbidity and mortality, in the context of a chronic or excessive alcohol intake [44]. It causes chronic liver failure, jaundice, coagulation disorders, marked asthenia, Ethanol encephalopathy, etc. More studies showed that the prognosis of alcoholic hepatitis is bad if the patient continues to consume alcohol, death occurring in 20–50% of cases, in about 28 days from the clinical onset of the disease [45–47].

Liver chronic inflammation, associated with hepatocyte necrosis, disturbs local homeostasis, and induces a regenerative, anarchist process, characterized by the production of an extracellular matrix (ECM) and especially of fibrillary collagen, with the onset of liver fibrosis. Liver fibrosis caused by excessive alcohol intake is progressive and ultimately leads to liver cirrhosis, a severe disease causing over one million deaths worldwide every year [15, 48].

The cellular and molecular mechanisms of ALD started to be well-known in the last years. In the first stage, alcohol induces liver steatosis because of the fatty acid reduction, activation of lipogenic enzymes, occurrence of reactive oxygen species (ROS) and some proinflammatory cytokines. All these lead to the onset of a local inflammatory process, where neutrophils are predominant. It was suggested that a high number of neutrophils is due to the activation of hepatocyte proteases, occurrence and increase of ROS. Proinflammatory cytokines, such as the tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6 and IL-8 are activated and secreted by the Kupffer cells and other inflammatory cells (neutrophils, macrophages), accumulated in the Kiernan spaces after a chronic alcohol intake [44, 49]. The presence of a local inflammatory process, even at a low level, may generate the onset of liver fibrosis, a more severe stage of excessive alcohol intake similar with liver damage of amyloidosis [50]. Liver fibrosis is the result of some qualitative and quantitative changes of the ECM. With its onset, the normal architecture of the liver is changed, leading to liver failure. Left untreated, fibrosis may progress to liver cirrhosis, most commonly progressing to severe liver failure and death [51–53].

During the liver fibrillogenesis process, there interfere the cells present in the liver. Thus, the hepatic stellate cells (Ito cells), under the influence of cytokines and chemokines synthesized by the inflammatory cells, trigger the transdifferentiation of the stellate cells in myofibroblasts. Proliferant myofibroblasts represent one of the main sources of ECM molecular excess (types I and III collagen), as well as of other proteins within the structure of pathological fibrous tissues [54–56].

In the process of liver fibrogenesis, there also intervene the Kupffer cells. They synthesize a multitude of strong cytokines, through which they perform the activation of liver stellate cells and their transdifferentiation in the myofibroblasts [57].

Our study showed that, in individuals with a chronic or excessive alcohol intake, there is an intense activity of the Kupffer cells and the transdifferentiation of liver stellate cells in the myofibroblasts.

Conclusions

Excessive alcohol intake causes important changes of the liver, including liver steatosis, hepatitis and even liver cirrhosis. Alcohol, directly or through its metabolites, induces molecular, infracellular and cellular changes that lead to hepatocyte necrosis, accumulation of inflammatory cells, synthesis and release of cytokines or other biochemical mediators. Biochemical mediators determine the transdifferentiation of liver stellate cells into myofibroblasts, a synthesis of high quantities of ECM, activation of fibrogenesis processes and, ultimately, to the onset of liver cirrhosis.

Conflict of interests

The authors declare that they have no conflict of interests.

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