UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA

DOCTORAL SCHOOL

PHD THESIS

ABSTRACT

CLINICAL-STATISTICAL, HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND WORK CAPACITY EVALUATION STUDY OF PATIENTS WITH VIRAL CHRONIC HEPATITIS

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Key words

Chronic viral hepatitis, hepatic fibrosis, alcohol consumption, comorbidities, work capacity

Chapter I. Liver histophysiology

Liver morphology. The liver is the largest gland in the body and the largest intra-abdominal parenchymal organ, with a weight ranging from 1200 to 1500 g . The liver is an amphicrine gland, has both an exocrine and an endocrine function. In the **Liver functional anatomy** we are talking about hepatic segment, which is due to the distribution of the portal pedicles and hepatic vein topography .

Liver development. The liver starts to develop in the 3rd week of pregnancy in the case of human embryos, as an endodermal bud, and it starts from the anterior section of the abdominal digestive tract towards the vitelline duct .

Hepatic functional units The basic structure is the classic hepatic lobule and it actually is the morpho-functional unit of the liver.Each classic hepatic lobule contains several "primary lobules". The "primary" lobules which contain a group of sinusoids fed by a single entry venule and whose termination is associated to a branch of the hepatic artery, have received another name, "hepatic microvascular subunits" HMS.

Chapter II. Hepatic fibrosis

Introduction. The hepatic fibrosis is the result of the acute or chronic hepatic lesions and it represents the accumulation of extracellular matrix, predominantly collagen fibbers, ultimately leading to the poor healing of the hepatic lesions, sometimes even to cirrhosis.

Clinical aspects of hepatic fibrosis. The fibrosis can accompany any chronic hepatic disease and it is characterized by the presence of the portal arthritic inflammation, and most worldwide cases of hepatic diseases are represented by chronic viral hepatitis.

Risk factors in the occurrence of hepatic fibrosis. As risk factors for severe fibrosis and increased risk of cirrhosis we mention: firstly, there is the increasing obesity, BMI>28kg/m, but there are also other factors, such as necroinflammatory activity, ALT>2x normal and/or AST/ALT>1, age, increased triglyceride levels, insulin resistance and/or diabetes mellitus and systemic hypertension .

Reversibility of hepatic fibrosis. It has been clearly shown that hepatic fibrosis can be reversible. The underlying cause of the hepatic diseases must be eliminated, either by eradicating HBV, HCV, HDV or by finding other ways to do so, depending on etiology.

Methods of assessing hepatic fibrosis. Percutaneous liver biopsy has been considered to be the best test for the assessment of hepatic fibrosis, but it is still an invasive method. There are many non-invasive tests which can be used as alternative to hepatic biopsy.

Treatment of hepatic fibrosis. There are several therapies: the anti-inflammatory therapies have treated the inflammation that generates the fibrogenic cascade, other treatments have tried to inhibit the cellular lesions or focus on activating the stellar cells, while others have targeted the collagen synthesis and matrix deposition.

Chapter III. Chronic hepatitis-Clinical and histopathological data

Chronic Hepatitis B Virus (HBV)

Worldwide, there are over 350 million carriers of the hepatitis B virus (HBV). **Geographical distribution and sources of exposure.** Half of the worldwide carriers of HBV live in extremely endemic regions such as the Southeast Asia (excluding Japan), China and a large part of Africa. **Clinical manifestations.** The main symptom is fatigue.

Histopathology of chronic hepatitis B. The lymphoplasmacytic periportal infiltrate is specific to the chronic hepatitis B virus, and, from the histological point of view, there are hepatocytes in the "matte glass" at the regular optic microscopy.
Exacerbations of chronic hepatitis B. Over time, spontaneous and repeated exacerbations lead to the worsening of histological lesions and of the fibrosis.
Serological diagnosis of acute or chronic hepatitis B. The hepatitis surface antigen (HBsAg) appears in the serum in approximately 2-10 weeks after the exposure to the virus and before the onset of symptoms or the rise of serum aminotransferases.

Chronic Hepatitis C Virus (HVC)

The hepatitis C virus is a major health problem in all the countries of the world . The hepatitis C virus was indentified only in 1989, the “non-A non-B virus”, and the first sample was highlighted in 1990.
Epidemiology. It is estimated that there are about 170-200 millions individuals infected worldwide, the highest prevalence of infection being in: Eastern Europe (10 millions), Southeast Asia (30-35 millions) and Africa (30-40 millions).
Clinical manifestations. The main symptom is physical and mental fatigue, but arthralgia, paraesthesia, myalgia may also occur. The clinical evolution is insidious and may slowly develop into cirrhosis.
The HCV-RNA tests are performed both in qualitative and quantitative terms. The qualitative tests give us information regarding the presence or absence of the virus and are more exact than the quantitative tests.

Chronic Hepatitis D Virus (HDV)

The hepatitis D virus (HDV) is a defective virus which requires the presence of the HBV virus in order to be transmitted. As **epidemiology,** it is the only virus belonging to delta virus, and concerning the **clinical or histological manifestations**, there are no aspects different from the regular hepatitis B. For the **diagnosis,** the indirect antibodies are determined, which are the immunoglobulin M, IgM -Ag HD and the total IgG antibody.

Liver biopsy techniques

The core needle hepatic biopsy has an important role in determining the etiology of the chronic hepatic disease, even if there are modern biochemical, serological, immunological and molecular tests which can do this, and even more, it determines both the inflammatory activity (grading) and the fibrosis / cirrhosis stage (staging).

Chapter IV. Importance and objectives of the study

A. Importance of the subject

The chronic hepatic diseases represent a major health problem worldwide. The prevalence of hepatitis C virus infection (HCV) is at about 3%, affecting approximately 170 million people worldwide.

B. Objectives of the study

Taking into account the complexity of the lesions in this doctoral thesis, we have set several objectives:
- the clinical, biological, evolutionary and work capacity analysis of a group of patients diagnosed with chronic viral hepatic diseases, which required the temporary decrease or interruption of physical activity and even ill health retirement;
- analysis of the social environment, of the consumption of hepatic toxins (alcohol), smoking and their contribution to the deterioration of the health condition and decrease or interruption of work capacity;

- analysis of the comorbidities and their influence on the decrease in work capacity;
- evaluation and grading of the histological lesions present in the liver of all patients under study;
- immunohistochemical study of the hepatocyte lesions, showing the severity of the hepatocyte necrosis lesions, the inflammation of the inflammatory cells, especially the T and B lymphocytes;
- highlighting the reaction of the Kupffer cells and of the dendritic cells in patients with chronic hepatitis;
- (yearly) assessment of the health of the persons included in the group.

Chapter V. Clinical-statistical study of patients with chronic viral hepatitis

Introduction. In order to further prevent the transmission of the disease and to stop its evolution to advanced liver fibrosis, liver cirrhosis and hepatocellular carcinoma, it is important to identity the diseases in a timely manner and to clinically manage them.

Material and methods. The study consisted of a group of 104 patients which arrived in the period January 2008 – December 2012 at the Medical Expertise Practice of Work Capacity – Slatina, which operates within the Olt National Pension House. In accordance with the Romanian legislation in force, all the patients from the group had the medical records to attest their health status issued by specialist doctors, internal medicine attending physicians or other categories of doctors from hospitals and polyclinics in Olt County or other counties or cities. For a correct analysis, we observed the following data for every patient: age, sex, background, type of hepatitis, the annual growth of viremia, transaminase evolution, date of record, occurrence date of disability, alcohol consumption, smoking or other toxicities, histopathological evaluation of hepatic lesions, presence of comorbidities.

Results. One of the first issues that we analysed was the sex of the patients registered with chronic hepatic diseases and with impaired work capacity. Most cases of chronic hepatitis have been reported in women (58 cases, representing a percentage of 55.77% of the whole group), compared to men (46 patients, representing 44.23%). The analysis of the patients depending on age showed that 14 patients (13.46%) are aged between 45 and 50; 43 patients (41.36%) are aged between 51 and 55 years; 42 patients (42.38%) are aged between 56 and 60 years, and just 5 patients (4.80%) are over the age of 61 years old. It can be seen that most of the patients with chronic hepatitis were aged between 51 and 60 years (85 patients, approximately 82%). Regarding the distribution of the group of patients depending on sex and age, we have found that men have been represented in all the age ranges, with a lower prevalence in all the age categories, except for the range of over 55 years, in which case they were numerically superior (27 men versus 20 women). In the medium age range, between 51 and 55 years old, the men / women ratio was of 2.07:1, indicating therefore a net prevalence of women.

Regarding the social environment of the patients included in the group, it was noticed that the majority of the subjects included in the study came from the urban area (73 patients representing 70.20%) compared to just 31 patients from the urban area (representing 29.80%), with a difference of over 40%. Distribution by age and place of origin. It is noticed that patients from the urban area dominate all the age groups. Also, the urban area was net superior both for the men and for the women ($p<0.0001$). The B viral infection was most common in the 51-55 year

group, while the C viral infection was predominant in the 56-60 year group. The B and C viral co-infection, as well as the association of hepatitis B with the D virus, was found only in the 51-60 year group. The only case of hepatitis of other cause than viral was found in a patient under 50 years old. The number of women was net higher for hepatitis B and C; however, the B+C viral co-infection was significantly more present in men (ratio of 2.33:1).

We also conducted a comparative study concerning the main behavioural factors associated with the viral infection. The majority of the patients (69.23%, respectively 72 patients) have denied alcohol or tobacco consumption; 15.38%, respectively 16 patients have admitted to both vices, just 1.93%, respectively 2 patients, having only the alcohol consumption as an aggravating factor. A total of 14 patients (13.46% of the whole group) were smokers. The noxious factors such as alcohol or tobacco consumption were predominant in the case of men 25 men reporting harmful behaviour, compared to only 7 women (ratio of 3.57:1).

Taking into account the etiology of the viral hepatitis, most smoking patients were infected with the C virus (6 patients, compared to 5 patients with B virus and three with B and C co-infection). The alcohol consumption was equally distributed among the B and C infections (one patient for each), while the association of both vices was predominant among B virus infected patients (7 patients, compared to 5 patients with viral hepatitis C and three with B and C co-infection) (Fig. 11). The only patient who did not have a viral cause for hepatitis also denied drinking alcohol, excluding, anamnestically at least, the alcoholic etiology of the hepatic impairment. Analysing the histogram of the annual variations of the GPT values, the distribution of the intermediately-increased values can be observed.The distribution of the values proves the variability in time of the transaminase values.

The most common comorbidities were those of the cardiological type, with a total of 38 patients; a total of 24 patients were also identified as having various affective problems.Four patients were also diagnosed with gastric or duodenal ulcer, while 5 patients were suffering from pulmonary diseases.

Discussion. Worldwide, the chronic hepatic diseases are an important burden for the health systems for the individuals, community and also a great economic burden. The chronic HCV infection can lead to cirrhosis, hepatic diseases in terminal stage and hepatocellular carcinoma.

The hepatic biopsy best assesses the degree of liver damage, the inflammatory activity and the stage of fibrosis, also confirming the hepatocellular carcinoma or identifying other diseases. However, the associated high cost, the invasiveness, the risk of complications limit its use in clinical practice, except for the cases in which the non-invasive baseline tests give inconclusive results.

Concerning the indirect fibrosis markers, transaminases, markers of synthetic liver function (albumin, bilirubin, prothrombin time) are used, or other indicators which refer to the stage of hepatic disease (platelet count). There are multiple studies combining all these parameters.

Chapter VI. Histological and immunohistochemical study of hepatic lesions in patients with chronic viral hepatitis

1.Histological aspects of hepatic lesions in chronic hepatitis

Introduction. Worldwide, HBV is the main factor which leads to HCC and generates approximately 50-55% of the cases of hepatocellular carcinomas .

Material and methods. Our study comprised 53 liver biopsies collected from the same number of patients diagnosed with chronic hepatitis. The patients were admitted with the diagnosis of post-viral chronic hepatitis in the Clinics of

Gastroenterology and Internal Medicine at the Craiova County Clinical Emergency Hospital, the Infectious Diseases Clinic at the "Victor-Babes" Clinical Hospital of Infectious Diseases in Craiova and the Medical Clinic at the "Filantropia" Municipal Clinic Hospital in Craiova. The biological material, immediately after being collected, was put in 10% neutral formalin fixation solution for 24 hours and sent to the pathological anatomy laboratory for paraffin inclusion, coloration and interpretation of results.

Histological methods used. The biological material was further processed according to the classical histological technique for paraffin inclusion. Histopatological interpretation of the results**.** The interpretation of the hispatological data was conducted by the anatomopathologists in accordance with the Metavir, Knodell și Knodell-Ishak scores.

Results. In our study, the inflammation, inflammatory processes, most often had a high intensity in the portal spaces and a low interlobular one. The hepatic fibrosis was very often noticed in patients with chronic hepatitis C. Most patients had microscopic aspects of moderated and severe fibrosis (stage 3-5) characterized by the presence of bridges of collagen fibbers with a port-port orientation. Also, the main hepatic lesions (necrosis, apoptosis, fibrosis and inflammatory infiltrate) can be associated in various stages.

Discussion.The inflammatory reaction in chronic hepatitis C is very complex. It is known that, after the virus infects the liver, the viral replication continues and the viral particles are continuously released into circulation. The inborn immunity provides an immediate response to the viral infection. It is certain that the inflammation of the liver is frequently associated with necrosis and hepatocyte apoptosis. These forms of damaging the hepatic cells initiate a sequence of events which are independent from the basic etiology of the inflammation and which can lead to hepatic fibrosis. The apoptotic bodies activate the resting hepatic stellate cells and the Kupffer cells, which in turn induce inflammatory and fibrogenic responses. Besides the necroinflammatory activity and the hepatic fibrosis, in our study we have also assessed one of the most serious lesions found in hepatitis. The hepatic fibrosis is the result of the scarring response to injuries. According to some studies, the hepatic fibrosis is frequently preceded by a chronic inflammation, and the persistence of this inflammation has been associated with progressive hepatic fibrosis and the development of cirrhosis. After an acute hepatic impairment, the parenchymal cells are regenerated and replaced by necrotic or apoptotic cells. This process is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). If the hepatic lesions persist, then the liver regeneration fails completely and the hepatocytes are replaced by abundant extracellular matrix (ECM), including fibrillated collagen. The main ECM-producing cells in the case of hepatic lesions are the hepatic stellate cells (HSC).

Actually, the mechanism by which the hepatitis C virus (HCV) causes progressive lesions of the hepatic parenchyma is unknown.

2. Immunoschemical aspects of hepatic lesions in chronic hepatitis

IHC methods used in order to begin the immunohistochemistry sequences, the sections were first deparaffinised in three successive xylene baths (for 15 minutes each) and then rehydrated by washing them in decreasing concentration alcohol (100%, 70%, 50%, for 15 minutes each). The sections were finally brought into distilled water in order to remove any trace of alcohol from the histological section.

Results. In our study, we have set to evaluate the inflammatory reaction present in the liver of the chronic hepatitis patients. Although there is a beneficial

response, a chronic inflammatory condition or an excessive inflammatory response may produce pathological effects.
In order to assess the inflammatory process present in the liver of the patients with chronic hepatitis C, we have qualitatively evaluated the presence of T-lymphocytes, B-lymphocytes, macrophages (Kuppfer cells) and hepatic dendritic cells using immunohistochemistry techniques. In the cases of moderate or advanced chronic hepatitis C, there were identified port-port and port-central fibrosis bridges, T-lymphocytes were present in these areas, accompanying the fibrosis process, which shows their contribution to the hepatic fibrosis process. The B-lymphocytes were less numerous than the T-lymphocytes. Also, there were found lymphoid follicles in the portal inflammatory infiltrate in some patients with chronic hepatitis C.In our study we have identified a large number of macrophages both in the gallbladder space and at the level of the hepatic lobe. The dendritic cells, also known as hepatic stellate cells (HSC), are present in the Disse perisinusoidal space, and, under pathological conditions, are capable of synthesis and secretion of extracellular conjunctive matrix, especially collagen fibbers. That is why many authors consider these cells to be responsible for the hepatic fibrosis. According to some studies, under pathological conditions, the ITO cells actively multiply, mobilize and produce collagen fibbers, being the main cells responsible for fibrillogenesis. We consider that the hepatic fibrillogenesis are much more complex, other cells also participating in the synthesis of the collagen fibbers, such as the fibroblasts and myofibroblast present in the gallbladder space.
Discussion. Using the immunohistochemistry techniques, our study highlighted the presence of numerous T-lymphocytes (CD3 +) and B-lymphocytes (CD20 +) in the portal inflammatory infiltrate, at the level of the port-port and port-central bridges resulted from the process of fibrillogenesis, as well as at the level of the hepatocyte cords. These microscopic aspects underline the importance of the lymphocytes in the healing process of the disease, but also in the pathological and development processes of the disease. The mechanisms which determine the inflammatory reaction of the liver are less elucidated because they are extremely complex. We consider that the presence of the hepatitis C virus in the liver represents the key element of triggering the inflammatory processes.
We have noticed in our study that the hepatic lesions intertwine and we think that the hepatocyte necrosis and hepatic inflammation are followed, in the absence of an effective treatment, by fibrosis processes as a normal response reaction of the body to an aggression. It has been also observed that the fibroblasts and the myofibroblasts appearing in the gallbladder space are heavily involved in the hepatic fibrogenesis.

Chapter VII. Study of work capacity in patients with chronic viral hepatitis

Introduction. The medical expertise of work capacity is a form of social-medical assistance which assesses through specific methods and techniques the work capacity of persons with different morphological and functional disorders for the social insurance benefits (social insurance). Invalidity is a medical-legal notion which expresses the particular status of a person insured through the public system of pension and other social security rights, who enjoy their rights according to the law in force .

Invalidity is quantified in relation to the possibility of carrying out activities related to daily and / or professional life, as follows: 1st degree of disability, total loss of working capacity, without the possibility of self-service; 2nd degree of disability,

total loss of working capacity, with the possibility of self-service, and the 3rd degree of invalidity with the loss of half of the working capacity.

Material and method. The study consisted of a group of 104 patients which had been medically assessed and retired on medical grounds at the Medical Expertise Practice of Work Capacity – Slatina, which operates within the Olt National Pension House. Most patients, which were initially retired as having the primary pathology for invalidity of viral cortical hepatitis, later developing other comorbidities or worsening of existing ones. The medical assessment of the work capacity of these patients was conducted annually for a period of 5 years, between 2008 and 2012.

Results. Out of the total of 104 patients, 46 are classified as 2nd degree of invalidity, and 58 as 3rd degree of invalidity (we have taken into consideration the degrees of invalidity at the end of the study period, respectively 2012). Out of the 46 patients in the 2nd degree of invalidity, 20 are classified in the 2nd degree of disability based on hepatic viral hepatitis as primary pathology for invalidity, which results in the classification in the degree of invalidity, the rest of 26 patients being classified in the 2nd degree of invalidity based on other diseases (comorbidities) (bipolar affective disorder, HDL operated with a Cauda equina syndrome).

Discussion. The chronic viral hepatitis, through their "silent" development, with a poor clinical picture, not having a development that affects the general condition of the patient, most of them manifesting only a slight fatigue, make the patients come to the doctor's in a late stage, many years after the initial infection, having a difficulty in obtaining a favourable therapeutic response to treatment. Most patients from the study had chronic hepatitis with moderate signs of clinical-biological activity, 3rd degree of invalidity, which shows a chronic, insidious evolution, most of the times without any changes in the yearly-conducted liver tests, which therefore shows a latent evolution of the disease over a long period of time (this includes the reduced alcohol or tobacco consumption or no consumption, reduced comorbidities or which do not influence the hepatic function, and an adequate lifestyle).

Chapter VIII. Conclusions

Our study consisted of a lot of 104 patients which arrived in the period January 2008 – December 2012 at the Medical Expertise Practice of Work Capacity – Slatina, which operates within the Olt National Pension House. The lot included only patients suffering from hepatic diseases as primary conditions, associated, or not, with other secondary ones. For a proper analysis, certain parameters were followed in these patients.

Most cases of chronic hepatitis have been reported in women (58 cases, representing a percentage of 55.77% of the whole group), compared to men (46 patients, representing 44.23%).

The analysis of the patients depending on age showed that most of the patients with chronic hepatitis were aged between 51 and 60 years (85 patients, approximately 82%).

Regarding the distribution of the group of patients depending on sex and age, we have found that men have been represented in all the age ranges, with a lower prevalence in all the age categories, except for the range of over 55 years, in which case they were numerically superior. Regarding the social environment of the patients, it was noticed that the majority of the subjects included in the study came from the urban area (73 patients representing 70.20%) compared to just 31 patients from the urban area (representing 29.80%), with a difference of over 40%.

The B viral infection was most common in the 51-55 year group, while the C viral infection was predominant in the 56-60 year group.

The histopathological and immunohistochemical study comprised a number of 53 liver biopsies collected from patients diagnosed with viral chronic hepatitis. The primary lesion which characterizes the chronic hepatic disease was represented by the death of hepatocytes, which is considered to be the triggering factor of the progression of the hepatic disease, by inflammation occurrence, development of fibrosis and, ultimately, the formation of the hepatic cirrhosis or malignant degeneration with the onset of the hepatocellular carcinoma.

The immunohistochemistry study revealed the participation of the immune system cells in the hepatic inflammatory reaction, we qualitatively evaluated the presence of T-lymphocytes, B-lymphocytes, macrophages (Kuppfer cells) and of hepatic dendritic cells at the level of the liver, which have contributed to the development of hepatic fibrosis.

Based on the clinical, histopathological, and immunohistochemical data, out of the total of 104 patients, 46 are classified as 2^{nd} degree of invalidity, and 58 as 3^{rd} degree of invalidity (we have taken into consideration the degrees of invalidity at the end of the study period, respectively 2012).

Out of the 46 patients in the 2^{nd} degree of invalidity, 20 are classified in the 2^{nd} degree of disability having viral chronic hepatitis as primary disabling condition, the rest of 26 patients being classified based on certain comorbidities associated with chronic hepatitis.