QUANTITATIVE ASSESSMENT OF THE HEPATIC LESIONS. CORRELATIONS WITH THE OPERATIONAL SCORES USED IN THE DIAGNOSIS OF CHRONIC HEPATITIS

SUMMARY OF THE PhD THESIS

PHD COORDINATOR
PROF. DR. IRINA-DRAGA CĂRUNTU

PHD STUDENT
NICUȘOR CORNEL STĂNCULEȚ

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# TABLE OF CONTENTS – PhD THESIS

## CHAPTER 1
**LIVER ARCHITECTURE AND PHYSIOLOGY**

1.1. GENERAL CHARACTERISTICS  
1.2. BLOOD SUPPLY  
1.3. LIVER ARCHITECTURE  
1.4. CORRELATIONS BETWEEN THE ARCHITECTURE OF THE LIVER AND THE FUNCTIONAL PROFILE OF THE HEPATOCYTES  
1.5. THE HEPATOCYTE  
1.6. THE LIVER SINUSOID  
1.7. INTRAHEPATIC BILIARY PATHWAYS  
1.8. LIVER PHYSIOLOGY  

## CHAPTER 2
**CHRONIC VIRAL HEPATITIS – ETIOPATOGENY AND PATHOLOGIC SUBSTRATE**

2.1. DEFINITION, HISTORY AND CLASSIFICATION  
2.2. ETIOPATHOGENY OF CHRONIC VIRAL HEPATITIS  
2.2.1. HEPATITIS VIRUS A  
2.2.2. HEPATITIS VIRUS B  
2.2.3. HEPATITIS VIRUS C  
2.2.4. HEPATITIS VIRUS D  
2.2.5. HEPATITIS VIRUS E  
2.2.6. HEPATITIS VIRUS G  
2.2.7. HEPATITIS VIRUS F  
2.2.8. TRANSFUSION ASSOCIATED VIRUS  
2.3. PATHOLOGIC SUBSTRATE IN CHRONIC VIRAL HEPATITIS  

## CHAPTER 3
**MOTIVATION AND OBJECTIVES OF THE PhD STUDY**  

## CHAPTER 4
**OPERATIONAL SCORES IN THE DIAGNOSIS OF THE CHRONIC HEPATITIS. SEMIQUANTITATIVE ANALYSIS**

4.1. INTRODUCTION  
4.2. MATERIAL AND METHOD  
4.3. RESULTS  
4.3.1. QUALITATIVE ASSESSMENT  
4.3.2. SEMI-QUANTITATIVE ASSESSMENT  
4.4. DISCUSSION  

## CHAPTER 5
**RELATIONSHIP BETWEEN KUPFFER CELLS – INFLAMMATORY LESIONS AND FIBROSIS IN CHRONIC HEPATITIS B AND C**

5.1. INTRODUCTION  
5.2. MATERIAL AND METHOD  
5.3. RESULTS  
5.3.1. QUALITATIVE ASSESSMENT  
5.3.2. QUANTITATIVE ASSESSMENT
This thesis is illustrated by 197 figures and 18 tables. The abstract includes a limited number from this total, while keeping the numbering in the thesis.
INTRODUCTION

The concept of viral chronic hepatitis appeared during the Second World War, when for the first time chronic diseases of the liver were described at the soldiers with jaundice episodes (Kalk, 1947).

Although considered at the beginning as an “intermediate stage” between acute hepatitis and liver cyrrhosis, with time chronic hepatitis rised numerous issues in definition and classification. The common denominator of the general definition is represented by the inflammatory lesions that persist for more than 6 months in the liver parenchyma and are associated with a series of biochemical and serologic modifications, which result from the structural alterations.

During the 50’s, the terms „chronic persistent hepatitis” – corresponding to a non-progressive variation of the viral hepatitis, with prolonged evolution and chronic active hepatitis (Smetana, 1954, Saint et al., 1953) are introduced. Although this new terminology can be found in the monography “The Chronic Hepatitis” edited in 1966, the first classification authored by an international group of hepatologists, which tries to correlate the histopathologic aspects of the liver biopsy with the prognosis of the disease, is published in 1968 (de Groote et al., 1968). The histopathologic criterion which differentiated the two forms is the periportal necrosis occuring in fragments, called ”piecemeal necrosis”.

The polymorph clinical and biological manifestations of this disease caused the apparition, during a large period of time, of numerous attempts of classification for the chronic hepatitis, based on various criteria.

The first paper that classifies chronic hepatitis (de Groote et al., 1968), regarded as a classic and quoted as reference point in the development of the research on hepatitis describes the two types, chronic persistent hepatitis (with possible benign prognosis) and chronic aggressive hepatitis – active (with possible severe prognosis) in correlation with the placement of the inflammatory infiltrate (Lefkowitch, 2007). The biopsies prove that, while the first type is defined by an inflammatory infiltrate consisting of mononucleate cells and limited to the portal space, the second type displays the expansion of the infiltrate beyond the edge of the portal space, in the limiting plaque (periportal) of the liver parenchyme (Lefkowitch, 2007).

In 1971, Popper and Schaffner took into account the lobular component in the form of lobular chronic hepatitis (Lefkowitch, 2007). The classification into chronic persistent hepatitis, chronic active hepatitis and chronic lobular hepatitis was applied for both viral and nonviral etiology and it dominated the terminology for over a quarter of a decade (Lefkowitch, 2007). This classification intended for a more precise association between the pathologic lesions and the prognosis of the disease. However, later prognostic connotations were recorded far more complex than the aspect of the lesions caught at a certain point in time (Lefkowitch, 2007).

Through the accumulation of important knowledge regarding the etiology, the pathogenesis, the clinical profile and the pathology of the chronic hepatitis, the old terminology became a confusion source, by using the same diagnosis (e.g. chronic active hepatitis), for diseases with different origins (viral, autoimmune, metabolic) or chronic persistent hepatitis/chronic active hepatitis (CPH/CAH) for the diagnosis of chronic viral hepatitis (Kenneth et al., 1995, Lefkowitch, 2007).

These reasons imposed the design and formulation of a new classification, based on etiology, because of the substantial differences between the clinical aspects, the prognosis and the treatment of the different types of chronic hepatitis – consensus achieved by the International Liver Research Association, (Desmet et al., 1994) and the World Gastroenterology Congress with result in the publication of the new terminology and classification of chronic hepatitis, based on etiologic criteria (Lefkowitch, 2007).

The pathologic exam is the one that confirms the intensity of the lesions and offers at the same time the definitory criteria for the classification of the diagnosis and its prognosis. In order to allow for a uniform appreciation of the intensity of the lesions noticeable during the microscopic exam, a series of diagnostic scores were introduced. From their multitude, the most used are $\Psi$ Knodell (Brunt, 2000), Metavir (Bedossa, Poynard, 1996) that can be applied only for HPC chronic hepatitis, Ishak score (Ishak, 1994) and Schauer system (Schauer, 1995) (Lefkowitch, 2007).

According to these scores, the chronic hepatitis are classified into three different severity stages: chronic hepatitis with mild activity, chronic hepatitis with moderate activity and chronic hepatitis with severe activity.
CHAPTER 3. OBJECTIVES AND MOTIVATION OF THE PERSONAL RESEARCH

The investigation of the biopsy specimen on the basis of semiquantitative assessment criteria (scores) contributes to the confirmation of the clinical diagnosis, through the pathologic assessment of the degree of development of the necroinflammation and fibrosis; moreover, it reveals the possible presence of associated pathologic processes and orientates the therapeutic intervention.

However, an unavoidable degree of subjectivity intervenes in the usage of the operational scores. Therefore, lately became more and more obvious the need for the implementation of distinct systems for the characterization of lesions in the liver, with the purpose to meet the requirements of the clinical perspective, which focuses predominantly on the therapeutic finality, but also of the researcher, in whose investigation the objective quantitative details are necessary.

In this context, the PhD research focused, in chronic B and C hepatitis:
- the assessment of the correspondence between the necroinflammatory activity and the fibrosis stage ascertained through the Ishak and Metavir scoring system and the analysis of the overlaps and differences between the two classification;
- evaluation of the kupfferian hyperplasia and lymphocytic proliferation and the correlation with the severity of the liver lesions, thus following the manner in which Kupffer cells, CD4+ and CD8+ T lymphocytes modulate the necro-inflammatory changes and the fibrosis specific for the morphologic substrate of chronic hepatitis;
- the design and implementation of an automated quantification algorithm for the lesions characteristic for fibrosis.

CHAPTER 4. OPERATIONAL SCORES IN THE DIAGNOSIS OF THE CHRONIC HEPATITIS. SEMIQUANTITATIVE ANALYSIS

4.1. INTRODUCTION

The scoring systems have unavoidably strengths and weaknesses in their main objective – the semiquantitative classification of the necroinflammatory activity and the staging of fibrosis. In time, numerous papers published relevant results supporting or contesting, through concrete data, the diagnostic and prognostic relevance of these scoring systems.

Our research is based on the morphologic image resulted from the semiquantitative evaluation of a significant group of liver biopsies performed in chronic hepatitis B and C. Starting from the quantification of the specific lesions, we focused on:
- the assessment of the correspondence between the necroinflammatory activity and the fibrosis stage ascertained through the Ishak scoring system in both hepatitis B and C;
- the analysis of the classification overlaps and differences of Ishak versus Metavir score.

At the same time, our analysis created the necessary support for the critical interpretation of the different classification systems, applicable in the assessment of the chronic hepatitis.

4.2. MATERIAL AND METHOD

The study group consisted of 953 cases of chronic viral hepatitis (202 cases with chronic hepatitis B and 751 cases with chronic hepatitis C), diagnosed in the Pathology Laboratory of the Clinical Hospital for Infectious Diseases „Sf. Parascheva” Iași, between 2008 and 2010.

Liver biopsy was performed on all the patients included in the study group through the percutane method, with the special Hepafix kit (B. Braun Melsungen AG). The fragments of hepatic tissue (dimensions: 10-25 / 1-1.4 mm) were fixed in formol and then processed according to the standard protocol of the Pathology Laboratory. The histology specimens were than stained with HE, trichrome Szekelly, and Gordon-Sweet colorations and were interpreted with the help of Ishak and METAVIR scores.

4.3. RESULTS

4.3.1. Qualitative assessment

In our group, the morphology of the chronic hepatitis ranged from mild to severe forms, some with a tendency towards cirrhosis. In the mild forms, the inflammatory infiltrate (formed by lymphocytes, macrophages, few plasma cells and rare neutrophils and eosinophils) was restricted to
the portobiliary space, the hepatic architecture being preserved. Various degrees of Kupffer cells hyperplasia was observed in both types of chronic hepatitis – B and C.

The following images illustrate some of the significant morphologic lesions (fig. 4.1-4.6).

**Fig. 4.1. Chronic severe hepatitis – micro and macrovesicular steatosis (HE, x 40)**

**Fig. 4.2. Chronic moderate hepatitis – Councilman bodies at the interface with the portal space (HE, x 40)**

**Fig. 4.3. Chronic moderate hepatitis – porto-portal fibrous bridges (TS, x 20)**

**Fig. 4.4. Chronic moderate hepatitis – important portal fibrosis with a tendency of expansion at the lobular level (TS, x 10)**

**Fig. 4.5. Chronic severe hepatitis – important portal lymphocytic infiltrate (HE, x 20)**

**Fig. 4.6. Chronic severe hepatitis – porto-portal bridges and destruction of the architecture of the liver (GS, x 20)**
The steatosis of the hepatocytes, more frequently macrovesicular but also microvesicular, with a more often disorganised topography was a common morphologic element.

4.3.2. Semi-quantitative assessment
For the 202 cases diagnosed with hepatitis B, the values assigned in the semiquantitative assessment with the application of the Ishak score for the necroinflammatory activity and fibrosis are presented in Table 4.1.

Table 4.1. Distribution of the cases with chronic hepatitis B in correlation with the NAI and fibrosis – Ishak score

<table>
<thead>
<tr>
<th>Necro-inflammatory activity</th>
<th>Total number of cases</th>
<th>NAI SCORE</th>
<th>Number of cases</th>
<th>FIBROSIS SCORE</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>mild</td>
<td>48</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SCORE 1-6</td>
<td></td>
<td>5</td>
<td>38</td>
<td>1</td>
<td>38</td>
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<td></td>
<td></td>
<td>6</td>
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</tr>
<tr>
<td>moderate</td>
<td>148</td>
<td>7</td>
<td>92</td>
<td>1</td>
<td>82</td>
</tr>
<tr>
<td>SCORE 7-10</td>
<td></td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>49</td>
<td>3</td>
<td>49</td>
</tr>
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<td></td>
<td></td>
<td>10</td>
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</tr>
<tr>
<td>severe</td>
<td>6</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SCORE 11-18</td>
<td></td>
<td>12</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

For the 751 cases diagnosed with hepatitis C, the values assigned in the semiquantitative assessment with the application of Ishak score (for the necroinflammatory activity and fibrosis) as well as the Metavir score are summarized in Table 4.2.

Table 4.2. Distribution of the cases with chronic hepatitis C in correlation with the Ishak score (NAI, fibrosis) and Metavir score

<table>
<thead>
<tr>
<th>Necro-inflammatory activity</th>
<th>Total number of cases</th>
<th>NAI SCORE</th>
<th>Number of cases</th>
<th>FIBROSIS SCORE</th>
<th>Number of cases</th>
<th>METAVIR SCORE</th>
<th>Number of cases</th>
</tr>
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<tbody>
<tr>
<td>mild</td>
<td>26</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>A1F1</td>
<td>23</td>
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<td>7-10</td>
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<td>1</td>
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<td>A2F1</td>
<td>3</td>
</tr>
<tr>
<td>SCORE 7-10</td>
<td></td>
<td>2</td>
<td>30</td>
<td>A2F1</td>
<td>30</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>606</td>
<td>A2F2</td>
<td>606</td>
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</tr>
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<td></td>
<td>4</td>
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<td>A2F3</td>
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<td></td>
<td>6</td>
<td>2</td>
<td>A3F3</td>
<td>28</td>
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<td></td>
<td></td>
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<td>A2F4</td>
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<td>A3F4</td>
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<tr>
<td>severe</td>
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<td>52</td>
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<td>8</td>
<td>5</td>
<td>1</td>
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<td></td>
</tr>
</tbody>
</table>

4.4. DISCUSSION
Beginning with the year 2000, the advances in the development of the biologic and imaging techniques lead to the introduction of new less invasive methods (Lefkowitch, 2007). These methods are based on the follow-up of the seric biochemical markers (Poynard et al., 2003, Lefkowitch, 2007) and the imagistic evaluation of the liver through elastography, in order to measure its rigidity, with a
correspondence in fibrosis (Collett et al., 2005, Castera et al., 2005, Ghany, Doo, 2005, Lefkowitch, 2007). However, biopsy remains a useful tool – not only for the confirmation of the clinic diagnosis but rather for the estimation of the prognosis and the choice (or not) of the antiviral therapy (Almasio et al., 2005, Lefkowitch, 2007).

The pathologic picture of the chronic hepatitis may vary considerably from case to case, but in essence these variations are due to the relationship between the three possible components: inflammatory reaction, fibrosis (or cirrhosis) and hepatocellular changes.

The morphologic features are generally similar to all forms of chronic, viral or other types of hepatitis. Nevertheless, there are some distinct elements, which we will discuss in correlation with our study. In chronic hepatitis B, the hepatocytes with “ground glass” cytoplasm were evident, appearance caused by a massive production of AgHBs in the hepatocytes which determines the proteic coating of the HBV to be accumulated in excess within the RER cisternae. The morphologic image for the hepatitis C was characterized by the presence of lymphoid aggregates in the portobiliary spaces – considered to be a specific signature (Lefkowitch, 2007) and by lesions of the biliary pathways.

Our study summarizes a morphologic analysis performed on almost 1000 liver biopsies investigated during a 3 years interval. The semiquantitative evaluation of the microscopic specimens was achieved on the basis of a previous rigorous instruction, the scores granted being periodically ascertained through an inter-observers diagnostic appraisal.

Within this context, our results create the premises for a three-directional discussion: the significance of the analysis on the correlation between the necroinflammatory activity and fibrosis, the motivation of the choice and application of a certain scoring system and the minimal condition necessary for the assessment of a liver biopsy.

- **Correspondences versus differences between the necroinflammatory activity and fibrosis**

The correlation between the necroinflammatory activity and the fibrosis is essentially the main element which permits a prognostic evaluation and, at the same time, an adequate therapeutic decision.

For chronic hepatitis B, our results reveal that the moderate necroinflammatory activity (score 7-10) involves simultaneously an extremely variable development of fibrosis, from the limited, restricted form (score 1) to the expanded form, characterized by fibrous bridges (score 4). On the contrary, for the mild and severe necroinflammatory activity, the correlation with a specific degree of fibrosis respects (in a certain manner) a better defined pattern. Thus, the mild form (score 1-6) associates fibrosis score 1 (mild), while the severe form (score 11-16) associates fibrosis score 3 and 4 (severe).

Conversely, in the chronic hepatitis C, the analysis of the correspondence necroinflammatory activity – fibrosis revealed a different pattern, with a wider variability. Firstly, the mild necroinflammatory activity (score 1-6) correlated with mild fibrosis (score 1), moderate (score 2) but also severe (score 3). Fibrosis score 1 was present in case of necroinflammatory activity score 2 but also in case of necroinflammatory activity score 6. At the same time, the necroinflammatory activity score 6 was associated with mild fibrosis (score 1), moderate (score 2) but also severe (score 3). Comparing the values of the scores, the development pattern of fibrosis is similar with the image present in moderate necroinflammatory activity (score 7-10) in hepatitis B. For the moderate necroinflammatory activity (score 7-10), the expansion degree of the fibrosis ranges from score 1 to score 6 – similar to the results obtained in mild necroinflammatory activity for hepatitis B – with the remark that the overwhelming majority of the cases (606 out of 672) presented fibrosis score 3 – which is the first step on the scale of severe fibrosis quantification. Moreover, the results obtained for the severe necroinflammatory activity (score 11-18) were similar to the ones in hepatitis B, associated with severe fibrosis – with the remark that in this class of the classification the severity degree was higher (score 4 and 5).

The comparison between the description of the necroinflammatory activity and fibrosis in hepatitis B versus hepatitis C offers, through the numeric values of the Ishak scoring system, accurate proofs, which support the aggressivity of hepatitis C, in whose patogeny fibrosis develops more quickly, even on the background of mild necroinflammatory activity.

Also, our results reveal that the necroinflammatory activity and fibrosis are not processes which progress in a consistent pattern. The extreme diversity of the fibrosis degree reflects, in essence, the biologic individuality that defines the response to the viral aggression.

- **Score system selection**

The final purpose of the scoring systems used in the interpretation of the liver biopsy, by the
evaluation of the degree and activity stage of the inflammatory disease and fibrosis, is to convey clear and precise information to the clinician (Lefkowitch, 2007). Consequently, the choice and usage of an operational scoring system must be founded on (Lefkowitch, 2007): 1) the existence of a consensus between pathologists and clinicians on the pathologic criteria quantified in the selected scoring system; 2) the concise and consistent formulation, in the pathology report, of the information about classification and staging; 3) the possibility to apply the obtained information in diagnosis and therapy.

On the basis of our experience in the semiquantitative evaluation of liver biopsies, both for hepatitis B and C, we use the Ishak scoring system. Our choice is supported by the fact that the wide range of numeric values attributed to the evaluation of necroinflammatory activity and fibrosis provides far more precise criteria for the appraisal of the degree of damage to the hepatic parenchyma at the time of the diagnosis. Supplementary to the Ishak scoring system, for the hepatitis C we perform at the same time a META VIR scoring system assessment, because it allows an appraisal of the entire histologic activity, with the addition of the interface hepatitis and the associated lobular necrosis components.

• Minimal requirements in the evaluation of a liver biopsy

Lever biopsy is a representative sample of the hepatic parenchyma. However, liver biopsy, regardless of its basic length or width, is only a finite segment from an organ with an immense potential diversity in the expression of disease (Lefkowitch, 2007). The pathologists obviously prefer larger samples for the interpretation (Demetris, Ruppert, 2003), and comparative data currently indicate that 20 mm long samples are necessary, which must include at least 11 portobiliary spaces in order to have an optimal specimen for grading and staging (Colloredo et al., 2003). In our study group, each microscopic specimen had between 3 and 12 portobiliary spaces, with an average of 6 to 8 – value that can support a correct diagnostic evaluation.

CHAPTER 5. RELATIONSHIP BETWEEN KUPFFER CELLS – INFLAMMATORY LESIONS AND FIBROSIS IN CHRONIC HEPATITIS B AND C

5.1. INTRODUCTION

In 1876, Karl Wilhelm von Kupffer described for the first time a highly represented cell population in the liver sinusoid capillaries, and he named it "Sternzellen" or stellate liver cells, according to their shape (Haubrich, 2004). At that time, it was believed that these cells are an intrinsic part of the endothelium of the liver sinusoids and, furthermore, that it derived directly from these cells (Haubrich, 2004). Kupffer cells are the main phagocytic and antigen presenting cells in the liver sinusoids, being at the same time important sources of cytokines with local chemotactic effect or with stimulatory action on the endothelial and Ito cells. Although standard light microscopy reveals the increase in the number of Kupffer cells in liver sinusoid and even their „agglomeration” in certain territories, the precise appreciation of the intensity of this phenomenon can be achieved only by supplementary exams, such as immunohistochemistry.

The purpose of our study is to evaluate the Kupfferian hyperplasia in viral chronic hepatitis with B hepatitis virus (HBV) and C hepatitis virus (HVC), respectively, and to correlate the Kupfferian hyperplasia with the severity of liver lesions, focusing on the modality in which Kupffer cells modulate the necroinflammatory events and fibrosis specific to the morphologic substrate of chronic hepatitis.

5.2. MATERIAL AND METHOD

The study group consisted of 71 cases of chronic viral hepatitis (subgroup 1 – 33 cases with chronic hepatitis B, subgroup 2 – 38 cases with chronic hepatitis C), diagnosed in the Histopathology Laboratory of the Clinical Hospital of Infectious Diseases “St. Parascheva” Iaşi.

The selection of the cases was performed on the basis of the histopathologic diagnosis established according to Ishak (in chronic hepatitis B and C) and META VIR score (in chronic hepatitis C), respectively, the main criterion being the classification into a severity stage in correlation with the mild, moderate, and severe necroinflammatory activity. Thus, subgroup 1 included: 10 cases with mild necroinflammatory activity (score 1-6), 17 cases with moderate necroinflammatory activity (score 7-10), and 6 cases with severe necroinflammatory activity (score 11-18), and subgroup 2: 11
cases with mild necroinflammatory activity (score 1-6), 18 cases of moderate necroinflammatory activity (score 7-10), and 9 cases of severe necroinflammatory activity (score 11-18).

The studied group was immunohistochemically investigated using an anti-CD68 antibody (clone KP1, Novocastra, Leica Biosystems), dilution ratio of 1:100, and the heat-induced epitope retrieval (HIER) technique. The quantitative evaluation was performed by two independent examiners, on 5 microscopic fields/case, at magnification x 200. After the quantification of the CD68 positive cells on all the examined areas, the median value obtained was associated with the following scoring system (Boisclair et al., 2001, Carotenuto et al., 2005, Tokin et al., 2011): < 10 CD68 positive cells – score 0; 10-49 CD68 positive cells – score 1; 50-99 CD68 positive cells – score 2; ≥100 CD68 positive cells – score 3. The t-Student test was used for the interpretation of the results.

5.3. RESULTS
5.3.1. Qualitative assessment

The qualitative evaluation of the distribution of the CD68 positive cells in the sinusoid capillaries allowed the identification of the following aspects:

- the presence of a reduced number of CD68 positive cells, localized mainly in the periportal sinusoidal spaces at the patients diagnosed with chronic liver disease with mild forms of activity;
- the tendency of extension to the entire liver lobule and, focally, the concentration of CD68 positive cells in certain “areas of hyperplasia” in the sinusoid capillaries, events closely correlated with the increase in the severity of the necroinflammatory lesions and in both portal and intralobular fibrosis.

The images in fig. 5.1 – 5.4 illustrate the distribution of Kupffer cells, immunohistochemically labeled, in the liver parenchyma.
5.3.2. Quantitative evaluation

The quantitative evaluation of the Kupffer cells is summarized in tables 5.3 and 5.4.

Table 5.3. Kupffer cells in correlation with Ishak scoring system (NIA, fibrosis) in chronic hepatitis B

<table>
<thead>
<tr>
<th>NIA</th>
<th>Cases (# total)</th>
<th>NAI score</th>
<th>Cases (#)</th>
<th>CD68+ cells (mean ± SD, score)</th>
<th>Fibrosis score</th>
<th>Cases (#)</th>
<th>CD68+ cells (mean ± SD, score)</th>
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<td></td>
<td>4</td>
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<td>24.54 ± 7.94 1</td>
<td>1</td>
<td>31.96 ± 14.67 1</td>
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<tr>
<td>moderate SCORE 7-10</td>
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<tr>
<td></td>
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<td>83.45 ± 28.44 2</td>
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<td>107.96 ± 18.87 3</td>
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<td></td>
<td>8</td>
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</tr>
<tr>
<td>SEVERE SCORE 11-18</td>
<td>6</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>5</td>
<td>126.45 ± 8.28 3</td>
<td>3</td>
<td>119.62 ± 15.62 3</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>12</td>
<td>1</td>
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<td></td>
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</tr>
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</table>

Table 5.4. Kupffer cells in correlation with the Ishak scoring system (NIA, fibrosis) and Metavir scoring system in chronic hepatitis C

<table>
<thead>
<tr>
<th>Cases (total number)</th>
<th>NIA (score)</th>
<th>CD68+ cells (mean ± SD, score)</th>
<th>Fibrosis score</th>
<th>CD68+ cells (mean ± SD, score)</th>
<th>Metavir score</th>
<th>Cases (#)</th>
<th>CD68+ cells (mean ± SD, score)</th>
</tr>
</thead>
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<td>11</td>
<td>mild</td>
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<td>33.51 ± 14.69 1</td>
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<td>4</td>
<td>30.64 ± 10.09 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>33.51 ± 14.69 1</td>
<td>A1F1</td>
<td>4</td>
<td>30.64 ± 10.09 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>33.51 ± 14.69 1</td>
<td>A1F1</td>
<td>4</td>
<td>30.64 ± 10.09 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>33.51 ± 14.69 1</td>
<td>A2F1</td>
<td>2</td>
<td>76.89 ± 18.36 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
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<td>A2F1</td>
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<td>76.89 ± 18.36 2</td>
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<td>A2F1</td>
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<td>76.89 ± 18.36 2</td>
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<td>A3F3</td>
<td>4</td>
<td>119.62 ± 15.62 3</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>moderate</td>
<td>86.79 ± 21.44 2</td>
<td>33.51 ± 14.69 1</td>
<td>A2F2</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>33.51 ± 14.69 1</td>
<td>A2F2</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td>33.51 ± 14.69 1</td>
<td>A2F2</td>
<td>3</td>
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<tr>
<td></td>
<td>9</td>
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<td>33.51 ± 14.69 1</td>
<td>A2F2</td>
<td>3</td>
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<tr>
<td></td>
<td>10</td>
<td>4</td>
<td>33.51 ± 14.69 1</td>
<td>A2F2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.3.3. Correlations between Kupffer cells and necroinflammatory activity – Ishak score

The t-student test revealed statistically significant differences for both types of chronic hepatitis between the number of Kupffer cells corresponding to the 3 degrees of necroinflammatory activities, in the comparison mild versus moderate (p < 0.001), mild versus severe (p < 0.001), and moderate versus severe (p < 0.001).

In the evaluation of chronic hepatitis B versus chronic hepatitis C no statistically significant differences were identified in the number of Kupffer cells corresponding to each degree of necroinflammatory activity: mild (p = 0.13), moderate (p = 0.70) and severe (p = 0.59).

5.3.4. Correlations between Kupffer cells and fibrosis – Ishak score

For both types of chronic hepatitis, the t Student test revealed the existence of significant differences in the number of Kupffer cells corresponding to the two degrees of fibrosis, mild versus severe (p < 0.001). For chronic hepatitis C, statistically significant differences were recorded also in the investigation of the number of Kupffer cells present in score 1 and 2 fibrosis versus score 3 (p <
0.001) (fig. 5.46) and score 4 fibrosis, respectively (p < 0.001) and also in score 3 fibrosis versus score 4 fibrosis (p < 0.001).

### 5.3.5. Correlations between Kupffer cells and METAVIR score

The correlation statistical analysis based on the Metavir score, applied only in chronic hepatitis C revealed significant differences (p < 0.001) in the number of Kupffer cells corresponding to the cases evaluated with A1F1/A1F2 score versus A2F1/A2F2 score versus A3F3 score.

### 5.4. DISCUSSION

#### 5.4.1. Kupffer cells and the necro-inflammatory activity – Ishak score

Kupffer cells exhibit an intense phagocytic activity in the parenchyma of the liver, acting as an filter for the antigen structures transported from the digestive tract through the portal vein. The phagocytic activity is supported by the synthesis of inflammatory cytokines, such as: IL-1, IL-6, IL-12, TNF, GM-CSF, CXCL9, CXCL10, MIP-1 (eng. macrophage inflammatory protein 1 alpha), and RANTES (eng. regulated on activation, normal T-cell expressed and secreted) (Cousens, Wing, 2000, Melgert et al., 2001, Kinoshita et al., 2010). Moreover, in viral liver pathology, the activated Kupffer cells express on their surface a series of molecules that provide them with similar features to an antigen-presenting cell, such as CD80 and CD40 (Kolios et al., 2006).

Currently, the idea of cellular heterogeneity within the Kupfferian cell population is increasingly supported, based not only on the classic relationship between the dimension and location in the liver sinusoids, according to which the periportal Kupffer cells are larger and more actively phagocytic as opposed to the ones situated around the centrilobular venule, who are smaller and mainly involved in the cytokine synthesis (Mc Guinness et al., 2000, Bilzer et al., 2006, Kinoshita et al., 2010).

Unfortunately, the data regarding the role of these cells in viral chronic hepatitis, with HVB and HVC are presently far from being elucidated (Cardoso et al., 2004, Szabo et al., 2007).

Our results based on the immunohistochemical labeling and the quantification of the Kupffer cells in chronic hepatitis B and C, respectively, in correlation with the necroinflammatory activity, revealed the statistically significant (p < 0.001) increase in the number of Kupffer cells, from the mild to moderate, and finally, to the severe necroinflammatory activity, respectively.

However, it is extremely interesting that the statistical analysis did not reveal significant differences between the number of Kupffer cells corresponding to each of the three different forms of necroinflammatory activity (mild, moderate, and severe) quantified in viral hepatitis B and C, respectively. From the point of view of the pathogenic mechanism, this result may indicate that the different evolution of the two types of hepatitis is not dependent of the Kupfferian hyperplasia, regarded strictly as numeric value, but rather of the specific behavior of the hepatocytes with respect to the pathogenic agent.

#### 5.4.2. Kupffer cells and fibrosis – Ishak score

Currently, the involvement of Kupffer cells in the activation of the Ito cells and in the fibrinogenic activity in the liver parenchyma is unanimously recognized (Maher, 2001, Ryder et al., 2004, Friedman, 2005, Heymann et al., 2009, Forbes, Parola, 2011). However, the molecular aspects of the interactions between the activated liver macrophages and the myofibroblasts are still incompletely known, representing a point of interest in the research directed toward the identification of new possible therapeutic targets in liver fibrosis (Tacke, 2012).

Recent studies showed that HVB chronic liver infection, even in cases exhibiting a mild necroinflammatory activity, is associated with the development of collagen synthesis in the sinusoidal and portal areas. This phenomenon is attributed to the reactivity of the Kupffer cells, which produce a series of pro-fibrinogenic cytokines, such as TGF-β1, even in mild forms of hepatitis (Li et al., 2012).

Moreover, it is unanimously accepted that patients with HVC have more severe fibrosis lesions as opposed to HVB chronic hepatitis patients (Lefkowitch et al., Tokin et al., 2011).

Consequently, in our research we focused on the search for a direct correlation between the intensity of the fibrosis lesions, developed concomitantly with the necroinflammatory activity and the hyperplasia of CD68+ cells.

Concretely, in the subgroup that included the cases with chronic hepatitis B:
- from the 10 cases with mild necro-inflammatory activity, 9 associated fibrosis score 1 and only one case –fibrosis score 2;
- from the 17 cases with moderate necro-inflammatory activity, 4 cases associated fibrosis score 1, 1 case associated fibrosis score 2 and 12 cases presented fibrosis score 3;
- from the 6 cases with severe necro-inflammatory activity, 4 cases associated fibrosis score 3 and 2 cases –fibrosis score 4.

In the subgroup that included the cases with chronic hepatitis C:
- from the 11 cases with mild necro-inflammatory activity, 4 cases associated fibrosis score 1, 4 cases presented fibrosis score 2 and 3 cases – fibrosis score 3;
- from the 18 cases with moderate necro-inflammatory activity, 2 cases associated fibrosis score 2, 12 cases associated fibrosis score 3 and 4 cases presented fibrosis score 4;
- all 9 cases with severe necro-inflammatory activity associated fibrosis score 4.

The evaluation of the fibrosis severity correlated with the necroinflammatory activity revealed more important lesions in chronic hepatitis C. As opposed to chronic hepatitis B, for the mild necroinflammatory activity there were more cases with score 2 fibrosis, as well as with score 3. For the moderate necroinflammatory activity, as compared to chronic hepatitis B, there were no cases with score 1 fibrosis, while score 4 fibrosis was identified in some cases. For the necroinflammatory activity, all cases showed fibrosis score 4, as opposed to chronic hepatitis B.

For each type of hepatitis, we recorded statistically significant differences between the number of Kupffer cells associated to the cases with fibrosis score 1 and 2, and to the cases with fibrosis score 3 and 4, respectively (p < 0.001). We also noted that the score value correlated with the expression of Kupffer cells increased from 1 to 3 in chronic hepatitis B, and from 1 to 2 in chronic hepatitis C, corresponding to mild and severe fibrosis, respectively. However, despite the above mentioned differences in the fibrosis score for the same intensity of the necroinflammatory activity, the statistical analysis revealed the lack of statistically significant differences between chronic hepatitis B and C, with respect to the number of Kupffer cells for the cases with fibrosis score 1 and 2, and cases with fibrosis score 3 and 4, respectively.

This negative result supports the idea that the development of fibrosis is an event not necessarily related to the Kupffer cells. The dramatic changes of the last 10-15 years concerning liver fibrosis are closely correlated with the deciphering of the extracellular matrix dynamics and ultrastructure, with the elucidation of the liver myofibroblasts biology and, last but not least, the integration of all these new elements in the complex process currently known as “connective tissue repair” (Friedman, 2008, Iredale, 2008).

It is also quite possible that the Kupffer cells intervene in the process of resolution of the liver fibrosis as well. Relatively recent hypotheses suggest that these cells could act through several mechanisms: directly, through the synthesis of certain proteases that degrade the extracellular matrix, or through the stimulation of other cells that produce these enzymes, among which it is possible that the stellate liver cells are included (Friedman, 2005).

Consequently, although investigated for a long time, the relationship between the Kupffer cells and liver fibrosis is still an open issue, its pro-fibrinogenic potential but, most of all, the anti-fibrinogenic one is far from being completely known.

5.4.3. Kupffer cells and Metavir score

Although the Metavir score ensures a specific and reproducible evaluation for C viral hepatitis (Standish et al., 2006), being largely used in the formulation of the histopathologic diagnosis, the review of the publications in the mainstream revealed the complete lack of the researches focused on the relationship between Kupffer cells and the severity of the lesions, in correlation with the Metavir scores in this type of hepatitis. To our knowledge, there is only one study that quantifies Kupffer cells and analyzes their dynamics with respect to the F (fibrosis) parameter of the Metavir score, in the pathologic context of chronic hepatitis induced by chronic alcohol consumption and of the fibrosis developed during the evolution of this entity (Chedid et al., 2004).

The mentioned study indicates a direct correlation between the expression of CD68, as marker for the Kupffer cells, and the severity degree of the disease, progressively from stage I to III (Chedid et al., 2004).
Within this context we believe that our data has a degree of novelty supported by the differences noticed in the comparison of the results obtained by the use of the two scores, Ishak and Metavir. In our opinion, the 3 classes of severity A1F1/A1F2, A2F1/A2F2, and A3F3 of the Metavir score reflect more accurately the evolution of the lesions, including through the correlation between the necroinflammatory activity and fibrosis, than the severity classes separately defined by necroinflammatory activity and fibrosis, respectively, in Ishak score. The most convincing argument is provided by the 4 cases that, according to Ishak score were classified as moderate NIA, score 10 and fibrosis score 4 but that according to the Metavir score were classified into a higher category of severity, through the A3F3 score. The statistically significant differences between the number of Kupffer cells (mean and SD) in all 3 classes of Metavir severity indicate the involvement of Kupffer cells in the increase of both inflammation and fibrosis, during the evolution of the severity of the lesions in hepatitis C.

CHAPTER 6. RELATIONSHIP BETWEEN CD4/CD8 T LYMPHOCYTES – INFLAMMATORY LESIONS AND FIBROSIS IN CHRONIC HEPATITIS B AND C

6.1. INTRODUCTION

Numerous studies are available in the literature focused on the investigation of the contents of the periporal and sinusoidal inflammatory infiltrate through immunohistochemical methods, in correlation with the etiology and the degree of necro-inflammatory activity (NIA) of the chronic viral hepatitis.

Within the inflammatory cell population in the liver parenchyma, the most numerous are the T lymphocytes (75%). In the structure of the T lymphocyte population, the ratio between the CD4+ and CD8+ cells is higher than 1.5 (Walewska-Zielecka et al., 2008). B lymphocytes are identified in a significantly lower proportion (15%) while the remaining 10% are represented by other cell types: macrophages and NK cells (Walewska-Zielecka et al., 2008).

Numerous studies showed that the specific consistent immune response of the host ensures the viral clearance, for the infection with both hepatitis virus B and C (Thimme et al., 2002, Rehermann, 2009, Fafi-Kremer et al., 2012). Consequently, the failure of the viral clearance, at least for the infection with hepatitis virus C is caused partially by an inadequate or insufficient specific immune response from the host (Thimme et al., 2012).

The aim of this study is to evaluate the T CD4+/CD8+ lymphocytes in chronic viral hepatitis with B and C virus and to correlate its expression with the severity of the lesions in the liver, thus following the manner in which CD4+/CD8+ lymphocytes modulate the fibrosis and the necro-inflammatory changes characteristic for the morphologic substrate of chronic hepatitis.

6.2. MATERIAL AND METHOD

The study group, the immunohistochemical protocol, the quantitative evaluation principles and the statistical instruments were presented in Chapter 5, which includes the research oriented on the Kupffer cells in correlation with the necro-inflammatory activity and fibrosis.

The differences that must be outlined are related to the antibodies used, namely anti-CD4 and anti-CD8, respectively. For each evaluated marker, the reported mean value resulted from the quantification of the cells present in 10 microscopic fields with high cellular intensity. At a magnification x 200.

6.3. RESULTS

6.3.1. Qualitative evaluation

The qualitative evaluation of the distribution of the T CD4+ and CD8+ lymphocytes in the liver parenchyma allowed for the identification of the following aspects:
- the presence of a small number of CD4+ and CD8+ lymphocytes located mainly in the periporal areas in the patients with mild forms of chronic liver disease;
- a tendency towards agglomeration of the CD4+ and CD8+ lymphocytes in the periporal areas, events closely correlated with the increase in the severity of the necro-inflammatory lesions and fibrosis both portal and intralobular.

The images in fig. 6.1 – 6.8 illustrate some of the results obtained in the study group investigated on the distribution of the immunohistochemically labeled CD4+ and CD8+ lymphocytes.
6.3.2. Qualitative evaluation

On the basis of the results obtained for each case, tables 6.4 and 6.5 summarize the relationship between the presence of the CD8+ and CD4+ lymphocytes, the intensity of the necro-inflammatory activity (established, according to the score, as mild, moderate and severe) and the fibrosis degree (appraised as mild for score 1-2 and severe for score 3-4) – Ishak score and table 6.6 – the correlation with Metavir scoring system.

For the entire group of cases with chronic hepatitis B, the numeric value for CD8+ was $70.16 \pm 29.50$, and for CD4+ - $95.46 \pm 22.55$. For chronic hepatitis C, the numeric value for CD8+ was $89.43 \pm 26.44$, and for CD4+ - $116.47 \pm 33.87$.

Fig. 6.1. CD4+ cells located in liver parenchyma (x 20)

Fig. 6.2. CD4+ cells located in portal space (x 40)

Fig. 6.3. CD4+ cells located in portal space (x 40)

Fig. 6.4. CD4+ cells located in portal space (x 40)

Fig. 6.5. CD8+ cells located in liver parenchyma (x 40)

Fig. 6.6. CD8+ cells located in liver parenchyma (x 40)
**Fig. 6.7.** CD8+ cells located in liver parenchyma (x 20)  
**Fig. 6.8.** CD8+ cells located in liver parenchyma (x 20)

**Table 6.4.** CD4+/CD8+ cells in correlation with Ishak scoring system (NIA, fibrosis) in chronic hepatitis B

<table>
<thead>
<tr>
<th>NIA</th>
<th>Cases (total #)</th>
<th>NIA score</th>
<th>Cases (#)</th>
<th>CD8+ cells (mean ± SD, score)</th>
<th>CD4+ cells (mean ± SD, score)</th>
<th>F score</th>
<th>Cases (#)</th>
<th>CD8+ cells (mean ± SD, score)</th>
<th>CD4+ cells (mean ± SD, score)</th>
</tr>
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<tbody>
<tr>
<td>mild SCORE 1-6</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>43.12 ± 8.27</td>
<td>76.65 ± 20.68</td>
<td>1</td>
<td>1</td>
<td>50.32 ± 13.83</td>
<td>84.28 ± 22.10</td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>6</td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>3</td>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>moderate SCORE 7-10</td>
<td>17</td>
<td>7</td>
<td>5</td>
<td>68.28 ± 11.40</td>
<td>97.44 ± 14.14</td>
<td>1</td>
<td>4</td>
<td>89.06 ± 28.61</td>
<td>119.00 ± 34.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>4</td>
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<td>5</td>
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<td>3</td>
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<td>5</td>
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<td></td>
</tr>
<tr>
<td>severe SCORE 11-18</td>
<td>6</td>
<td>11</td>
<td>5</td>
<td>120.23 ± 20.04</td>
<td>121.25 ± 18.08</td>
<td>3</td>
<td>3</td>
<td>121.25 ± 18.08</td>
<td>119.00 ± 34.55</td>
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<tr>
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<td>12</td>
<td>1</td>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
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<td></td>
<td>3</td>
<td>1</td>
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</tr>
</tbody>
</table>

**Table 6.5.** CD8+/CD4+ cells in correlation with Ishak scoring system (NIA, fibrosis) in chronic hepatitis

<table>
<thead>
<tr>
<th>Cases (total #)</th>
<th>NIA score</th>
<th>Cases (#)</th>
<th>CD8+ cells (mean ± SD, score)</th>
<th>CD4+ cells (mean ± SD, score)</th>
<th>F score</th>
<th>Cases (#)</th>
<th>CD8+ cells (mean ± SD, score)</th>
<th>CD4+ cells (mean ± SD, score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>mild SCORE 1-6</td>
<td>2</td>
<td>1</td>
<td>60.32 ± 13.01</td>
<td>82.14 ± 16.24</td>
<td>1</td>
<td>1</td>
<td>63.78 ± 16.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>5</td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
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<td></td>
<td></td>
<td>2</td>
<td>2</td>
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</tr>
<tr>
<td>18</td>
<td>moderate SCORE 7-10</td>
<td>7</td>
<td>5</td>
<td>91.03 ± 16.50</td>
<td>112.19 ± 13.15</td>
<td>2</td>
<td>2</td>
<td>63.78 ± 16.99</td>
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<td>5</td>
<td></td>
<td></td>
<td>3</td>
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<td></td>
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<td>4</td>
<td></td>
<td></td>
<td>3</td>
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<td>4</td>
<td></td>
<td></td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Severe SCORE 11-18</td>
<td>11</td>
<td>5</td>
<td>121.81 ± 9.65</td>
<td>166.89 ± 8.76</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>4</td>
<td></td>
<td></td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

The correlation statistical analysis revealed statistically significant differences between the number of CD8+ and CD4+ T lymphocytes both for group 1 – chronic hepatitis B and for group 2 – chronic hepatitis C (p < 0.0001).
Table 6.6. CD4+/CD8+ cells in correlation with Metavir scoring system in chronic hepatitis C

<table>
<thead>
<tr>
<th>Cases (#total)</th>
<th>Metavir score</th>
<th>Cases (#)</th>
<th>CD8+ cells (mean ± SD, score)</th>
<th>CD4+ cells (mean ± SD, score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>A1F1</td>
<td>4</td>
<td>60.33 ± 13.02</td>
<td>82.15 ± 16.25</td>
</tr>
<tr>
<td></td>
<td>A1F1</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1F2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>A2F1</td>
<td>2</td>
<td>87.90 ± 15.23</td>
<td>113.32 ± 12.38</td>
</tr>
<tr>
<td></td>
<td>A2F2</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>A2F2</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2F2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3F3</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>A3F3</td>
<td>9</td>
<td>115.72 ± 15.35</td>
<td>148.92 ± 30.33</td>
</tr>
</tbody>
</table>

Similarly, there were significant differences in the correlation CD8+ and CD4+ T lymphocytes, respectively, in chronic hepatitis B versus chronic hepatitis C (p = 0.005 and p = 0.002, respectively)

6.3.3. Correlations between CD4/CD8 lymphocytes and NIA – Ishak score

For both types of chronic hepatitis, the t-Student test revealed significant differences between the number of CD4+ cells corresponding to the 3 degrees of necro-inflammatory activity, in the comparison mild versus moderate (p < 0.0001), mild versus severe (p < 0.0001) and moderate versus severe (p < 0.0001).

For the number of CD8+ cells, in chronic hepatitis B there were significant differences between NIA mild versus moderate (p=0.01), mild versus severe (p=0.0007) and moderate versus severe (p=0.02). Similar results were recorded in chronic hepatitis C, with a high degree of statistic significance (p < 0.0001) between the 3 types of NIA.

6.3.4. Correlations between CD4/CD8 lymphocytes and fibrosis – Ishak score

For both types of hepatitis, statistically significant differences were recorded between CD8+ and CD4+ T lymphocytes, respectively, and the form of mild versus severe fibrosis (in all cases p < 0.0001, with the exception of chronic B hepatitis, CD4+ T lymphocytes in mild versus severe fibrosis, were p = 0.001).

6.3.5. Correlations between CD4/CD8 lymphocytes and METAVIR score

For chronic hepatitis C, evaluated through the Metavir score, the highly statistically significant differences (p < 0.0001) were recorded in the comparative evaluation of the CD8+ T lymphocytes with respect to the scores A1F1+A1F2 versus A2F1+A2F2, A2F1+A2F2 versus A3F3 and A1F1+A1F2 versus A3F3, as well as in the comparative evaluation of the CD4+ T lymphocytes with respect to the scores A1F1+A1F2 versus A2F1+A2F2 and A1F1+A1F2 versus A3F3, for A2F1+A2F2 versus A3F3 recording p = 0.001.

Also, there were significant differences between the number of CD8+ versus CD4+ cells corresponding to the cases evaluated with the scores A1F1/A1F2 (p = 0.0002), A2F1/A2F2 (p = 0.001) and A3F3 (p = 0.0008)

6.4. DISCUSSION

6.4.1. The role of T lymphocytes in the pathogeny of chronic hepatitis

Through its functional complexity based on the particular immune microenvironment, the liver has the ability to „tolerate” certain antigen structures or to initiate an immune response, especially against the structures of the hepatic viruses.

The liver parenchyma is characterized by three constant presence of a chronic inflammatory infiltrate both at portal and sinusoidal level. The portal inflammatory cell population is dominated by the presence of CD4+ T cells, while the B and CD8+ T cells are predominantly concentrated in the
liver sinusoids, whereas in the porto-biliary spaces are placed at the periphery (Walewska-Zielecka et al., 2007). Moreover, the infection with hepatitis virus C is sometimes associated with the development of secondary lymph nodes in the portal space, their presence being significantly more uncommon in the patients with chronic liver infection induced by hepatitis virus B (Murakami et al., 1999).

6.4.2. Lymphocytic populations in the liver parenchyma

During the documentation on this subject we identified very few numerical data reports resulted from the quantification of the lymphocytic population in the liver parenchyma (Mariani et al., 1984, Onji et al., 1988, Walewska-Zielecka et al., 2007, Carmack et al., 2008) and, consequently, the relative absence of correlations between the number of lymphocytes and the degree of severity of the disease, established through the scoring systems.

Within this context, we believe that our study represents an important contribution to the completion of the morphologic profile for the chronic hepatitis, from the point of view of the numerical characterization of the lymphocytic inflammatory infiltrate.

We must underline the fact that the numerical values obtained for both lymphocytic subtypes were smaller in chronic hepatitis B as opposed to chronic hepatitis C, with statistically significant differences, result that confirms through quantitative objective criteria (numbers) the morphologic differences described in the qualitative characterization of chronic hepatitis B and C.

For both types of chronic hepatitis we identified both subtypes of T lymphocytes, the global numerical values (mean ± SD of CD4+ T lymphocytes (95.46 ± 22.55 in chronic hepatitis B and 116.47 ± 33.87 in chronic hepatitis C) being larger as opposed to those of CD8+ T lymphocytes (70.16 ± 29.50 in chronic hepatitis B and 89.43 ± 26.44 in chronic hepatitis C), the differences being statistically significant.

On the basis of our results, we believe that the numerical values corresponding to the CD4+ T lymphocytic subtypes reflects the process of lymphocytic proliferation, which supports a benefic immune response developed by the host – the wrong CD4+ reaction being accompanied by an increase in the viremia within a few months from its apparent control (Gerlach et al., 1999, Nascimbeni et al., 2003), and associated with the chronicization of the disease.

6.4.3. CD4/CD8 lymphocytes and necro-inflammatory activity – Ishak score

Our results, based on the IHC labeling and the quantification of CD8+ T lymphocytes in chronic B hepatitis, with respect to the necro-inflammatory activity, revealed the statistically significant increase of their number between necro-inflammatory activity mild versus moderate (p = 0.01), mild versus severe (p = 0.0007) and moderate versus severe (p = 0.02). Concomitantly, the CD 4+ T lymphocytes presented also differences highly statistically significant between the 3 degrees of necro-inflammatory activity (p < 0.0001). Similar results were recorded in chronic hepatitis C for both lymphocytic subtypes, with a high degree of statistical significance (p < 0.0001). This data proves that the progressive proliferation of both lymphocytic subtypes is directly correlated with the gradual severity of the lesions in the liver reflected through the necro-inflammatory activity determined in evolution, through the values of the Ishak score, as mild, moderate and severe.

The absence of statistically significant differences between the CD4+ and CD8+ T lymphocytic population, in chronic B and C hepatitis, for the case of mild necro-inflammatory activity, reveals the relatively similar beginning, from the point of view of the pathology. However, later, in the lesions characteristic for the severe necro-inflammatory activity, the significant differences are absent in the CD8+ lymphocytic population. The interpretation of this data, taking into the account the fact that the clinic evolution of viral hepatitis C is much more aggressive as opposed to B chronic hepatitis is based on the functionality of the lymphocytes and not on their number – thus the CD8+ T lymphocytes cytotoxicity represents only a piece from the pathogenic mechanism of the lesions specific for hepatitis C.

6.4.4. CD4 / CD8 lymphocytes and fibrosis – Ishak score

In our research we focused on the identification of a direct correlation between the intensity of the fibrosis lesions, developed concomitantly with the necro-inflammatory activity and the CD4+ / CD8+ expression.
The fact that both in chronic B and C hepatitis the two lymphocytic subtypes differ statistically significant between the mild and the moderate fibrosis indicates the correlation between the number of lymphocytes and the intensity of the fibrinogenic activity.

The comparative analysis of the lymphocytic subtypes in chronic hepatitis B versus C revealed the presence of significant differences for CD4+ and CD8+ T lymphocytes in the severe fibrosis. These numerical results coincide with the important fibrinogenesis specific for chronic hepatitis C and suggests the role of the CD4+ T lymphocytes in the initial stage.

6.4.5. CD4/CD8 lymphocytes and Metavir score

The results obtained in the evaluation of CD4+ and CD8+ T lymphocytes in chronic hepatitis C in correlation with the grading of the lesions according to the Metavir score confirmed the data obtained in the statistical analysis applied with respect to the Ishak score. Essentially, the data indicated the presence of a correlation between the two lymphocytic subtypes and the intensity of the lesions, as well as the value of the ratio between CD4+ and CD8+ separately for each severity class.

CHAPTER 7. COMPUTERIZED MORPHOMETRY IN THE EVALUATION OF THE LESIONS OF FIBROSIS – DESIGN AND IMPLEMENTATION OF AN OPERATIONAL INSTRUMENT

7.1. INTRODUCTION

The inflammatory infiltrate, the piecemeal, bridging necrosis and fibrosis are the morphologic lesions that characterize the hepatitis. On the basis of these lesions identified from a qualitative point of view and with the use of unanimously accepted scores (Knodell, Ludwig, METAVIR), which allow for a semi-quantitative evaluation, the pathologist establishes the evolution stage, the severity/aggressivity degree of the disease and, possibly, determines the viral etiology.

Currently, a modern trend in international scientific research is the development of quantitative analysis techniques (or of interactive and/or automated morphometry) in order to obtain more accurate and objective information on the extent of the liver damage in hepatitis and cirrhosis.

In the last 3 decades, the literature comprises a small number of papers (under 30) dedicated to this subject (Manabe et al., 1993, Kage et al., 1997, Duchatelle et al., 1998, Pilette et al., 1998, Masseroli et al., 2000, O’Brien et al., 2000, Bedossa et al., 2003, Dioguardi et al., 2005).

On the basis of the reports in the literature oriented on this direction, our objective was represented by the design and implementation of an automated quantification algorithm of the specific fibrosis lesions that could be applied in chronic hepatitis B and C.

7.2. MATERIAL AND METHOD

7.2.1. Microscopic specimens, digitized images

The investigated material consisted of 16 microscopic specimens from liver biopsies, with trichrome Szekely colorations, corresponding to 8 cases of chronic hepatitis B and 8 cases of chronic hepatitis C.

In the selection of the microscopic specimens for computerized morphometry we aimed for the illustration of the evolution in the fibrosis lesions, reflected through the classification into different severity classes, established according to Ishak score.

The microscopic specimens were used in the creation of correspondent digitized images, through the facilities provided by the KS400 environment (Kontron Elektronik, 2002) that exists in the laboratory of the Department of Histology at the University of Medicine and Pharmacy “Grigore T. Popa” Iași.

The application of computerized morphometry was performed on the obtained digitized images. The design and implementation of this application resulted after the integration of this study in the research direction followed by the staff at the Department of Histology.

7.2.2. Digital image processing

The digital images obtained were processed in order to emphasize the visibility of certain morphologic elements of interest, through operations of brightness and contrast adjusting, filtering and noise reduction. The mathematical support of these operations is presented briefly below, according with
the specific concepts on which computer vision is founded (Shapiro, Stockman, 2001, Gonzales, Woods, 2002, Căruntu, 2003).

7.2.3. Segmentation of digital images

In the language specific for image processing, the morphologic entities represent regions in the digital image. The regions could be defined as groups of pixels characterized by a certain degree of homogeneity with respect to a certain feature: grey (or colour) level, or the module of the gradient for the grey level function (Căruntu, 2003). The operations through which the automated separation of regions (with similarities) from the digital image is performed is called segmentation. When the separation refers to only one type of regions, the result of the segmentation is a binary image where the pixels have either value 1 (white) if they are placed (from the point of view of location) within the regions taken into account or value 0 (black) if they are situated outside these regions (Căruntu, 2003). Only those pixels were selected with colour coordinates that fell within a parallelepiped with the tips given by \( L_R, L_G, L_B \) and \( H_R, H_G, H_B \), respectively from within the RGB cube. Thus, through the selection of the threshold values and, implicitly, through the delination of a specific range for each colour (between 0 and 255) we determined which of the morphologic elements in the image, corresponding to a certain RGB domain, are kept or not in the resulted image.

7.3. RESULTS

7.3.1. The design of HEPAT macro

The HEPAT macro was designed through the direct input of the instructions, without appealing to the dialogue boxes – thus reflecting a professional exploitation of the KS400 environment, on the basis of the usage experience developed throughout the PhD stage.

7.3.2. Quantification of fibrosis

The percentual numeric values of the fibrosis areas occupied in the total surface of the biopsy reveal a progressive increase correlated with the degree of fibrosis. For chronic hepatitis B, the minimum value corresponding to fibrosis score 1 was 2.6\% and the maximum value, corresponding to fibrosis score 4, was 12\%. For chronic hepatitis C, the minimum value, corresponding to fibrosis score 1 was 3.1\% and the maximum value, corresponding to fibrosis score 4, was 21.5\%.

Table 7.4 summarizes the percentual numeric information corresponding to the 4 degrees of fibrosis comparatively for chronic hepatitis B and C.

The comparison between the percentual values of the fibrosis areas occupied in the total surface area of the biopsy could not be performed through the instruments specific for statistical analysis, because of the small number of cases investigated for each fibrosis degree (2 cases).

However, the numbers indicate small differences in the percentage occupied by fibrosis in the cases evaluated with fibrosis score 1 and 2, between chronic hepatitis B and C, respectively, moderate differences for the cases with fibrosis score 3 and considerable differences, of over 4,5\%, for the cases with fibrosis score 4.

<table>
<thead>
<tr>
<th>Fibrosis score</th>
<th>Dimension of fibrosis (% total area of biopsy)</th>
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<tbody>
<tr>
<td></td>
<td>Chronic hepatitis B</td>
</tr>
<tr>
<td>1</td>
<td>2.57</td>
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<tr>
<td>2</td>
<td>3.50</td>
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<tr>
<td>3</td>
<td>4.90</td>
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<td>4</td>
<td>10.75</td>
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</table>

7.4. DISCUSSION

7.4.1. The value of computerized morphometry in the evaluation of liver fibrosis

The clinical practice on liver pathology reveals more and more that the evaluation of fibrosis is an extremely important element for the assessment of the evolution and prognosis for the chronic
hepatitis. In the normal status, the liver parenchyma has approximately 5.5 mg/g of collagen and in cyrrhosis lesions – 30 mg/g (Rojkind, Ponce-Noyola, 1982). In the evolution of various pathologic processes different quantities are deposited, according to the severity degree of the disease.

Unfortunately, the operational scoring systems in the grading of liver lesions – including fibrosis have limits that become more and more obvious. Consequently, the efforts of the specialists are oriented lately towards the identification and implementation of an adequate method for measurement. There were proposed biochemical methods, based on the measurement of the amount of collagen in tissue homogenates (Lee et al., 2005).

However, this type of methods require the destruction of the harvested tissue fragment, so the access to other information, through the investigation of the liver biopsy is no longer possible – the pathologic exam is compromised (Standish et al., 2006).

The computerized morphometry studies published in the mainstream cover numerous diagnosis entities in liver pathology (Manabe et al., 1993, Kage et al., 1997, Dukatelle et al., 1998, Filette et al., 1998, Masseroli et al., 2000, O’Brien et al., 2000, Bedossa et al., 2003), and report from 1-4% fibrous tissue in normal liver to 15-35% in cyrrhosis. Some of these studies focused on the correlation of the results from the measurement of fibrosis, obtained through computerized morphometry, with the scores that define different stages.

The implementation of the computer-assisted image analysis for the evaluation of fibrosis through computerized morphometry is a difficult process, with opportunities directed predominantly towards research and less towards diagnosis. The interest research groups advocate the uniformization of the working methodology, so that the computerized morphometry can be really used in the evaluation of liver fibrosis (Lazzarini et al., 2005, Hui et al., 2004, Wright et al., 2003).

Hence, any detail is important from the technical point of view (the stability of the light source, the characteristics of the camera, the segmentation threshold) and also from the medical – pathologic one (the size of the liver biopsy, the thickness of the sections, the coloration techniques, the elimination of normal collagen structures, without implication in the fibrosis process) (Wright et al., 2003).

In our investigation we used microscopic specimens with trichrome Szekelly coloration that allowed for a good identification of the collagen present in the fibrosis areas, through the thresholding segmentation operation. The value range in the RGM system (red – green – blue), determined through successive trials on one microscopic specimen was later introduced in the text of the macro HEPAT and applied in the computerized analysis of all the other microscopic specimens.

Consequently, we believe that a trichrome coloration of the green light type well performed allows for the repetitive application of a computerized morphometry algorithm. The results obtained in our study open real perspectives in the automated quantification of the fibrosis lesions, the actual validation requiring an extended investigation group.

7.4.2. Design and implementation of automated quantification instruments. Dimensions and scores

The designed and implemented macro HEPAT allowed the acquirement of valuable numerical results mainly through their correspondence to different stages of severity in the fibrosis process developed in chronic hepatitis B and C. This approach is relatively absent in the mainstream publications, so there are very few studies to which we can relate – a fact that reflects, essentially, the originality element in our investigation.

Our results are important as contribution to the state of knowledge for they offer comparisons between the fibrosis area occupied in the entire area of the liver biopsy from chronic hepatitis B and C. to our knowledge, on the basis of the review of the literature oriented on this subject, the only values of fibrosis percentage reported in correlation with the scores are 1.9% for the normal liver, 3% for fibrosis score 1, 3.6% for fibrosis score 2, 6.5% for fibrosis score 3, 13.7% for fibrosis score 4, 24.3% for fibrosis score 5 and 27.8% for fibrosis score 6 (Standish et al., 2006) – the measurements being performed on microscopic specimens stained with Sirius Red. Although our aim was not to evaluate the areas of inflammatory infiltrate, simultaneously with the fibrosis areas, this type of investigation can be achieved – if the liver biopsies are immunohistochemically stained, thus ensuring the possibility for automated identification of the macrophages and lymphocytes, through coloring characteristics.
On the basis of our experience, we believe that all these results indicate the necessity for the development of computerized morphometry techniques and their implementation in the current practice. Without the evaluation of the qualitative architectural transformations (fibrous septa between the portal spaces, bridging fibrosis, centro-portal, the nodes) on which the numerical value of the scores is based, the computerized morphometry ensures the accurate measurement of the fibrosis area. The two methods do not exclude one another, but must be seen as complementary instruments in the evaluation of the liver biopsy.

We must underline as an important limit the fact that the identification of collagen, which allows for the delineation and measurement of the collagen area and the recording of these measurements as fibrosis percentage, or the collagen percentage area in correlation with the total area of the biopsy, is performed through a segmentation operation which requires very well defined values of the threshold in order to ensure reproducibility; this operation is laborious, takes time and a very good knowledge of the pathologic elements characteristic for the liver pathology, the elimination of the collagen structures without signification for the morphologic substrate of the disease being mandatory. As a major advantage we must stress the fact that computerized morphometry does not interfere with other evaluations necessary for the pathologic exam, the biological product being preserved.

CHAPTER 8. CONCLUSIONS

1. The comparison of the image for the necro-inflammatory activity and fibrosis in hepatitis B versus C provides, through the numerical values of the Ishak score, real evidence that support the aggressivity of hepatitis C, in whose pathogeny the development of fibrosis is quicker, being initiated even on the background of mild necro-inflammatory activity. The actual values of Ishak score indicate that the necro-inflammatory activity and fibrogenesis are processes that do not happen in a correlated model. The extreme variability of the degree of fibrosis reflects, essentially, the biologic indivduality that defines the response to the viral aggression.

2. The advantages of the application of Metavir score in chronic hepatitis C are counterbalanced by the possibility of assigning an identical score for lesions with different intensity.

3. The progressive hyperplasia of Kupffer cells simultaneous with the intensity of the necro-inflammatory activity and the extent of the fibrosis lesions, but without statistically significant differences between chronic hepatitis B and C indicate that the progression of the lesions specific for chronic hepatitis C is influenced by the different behavior of the Kupffer cells and not by their actual number.

4. The numerical profile of the CD4+ T lymphocytic population indicates the similar implication in the necro-inflammatory lesions from the intiation of chronic hepatitis B and C, while the relationship with the fibrosis lesions is confirmed only for the intitial stages of chronic hepatitis C.

5. The numeric profile of CD8+ T lymphocytic population, without statistically significant differences between chronic hepatitis B and C reflects the fact that the cytotoxicity of CD8+ T lymphocytes represents only a piece in the pathogenic mechanism of the lesions specific for hepatitis C.

6. The assessment of fibrosis through computerized morphometry offers objective numerical information that reflect with high accuracy the degree of damage in the liver parenchyma, exceeding the information obtained through score values. The computerized morphometry evaluation does not exclude the application of the semi-quantitative scores, the two methods being complementary instruments in the evaluation of liver biopsy.

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