Chapter 12

Future Aspects of Liver Biopsy: From Reality to Mathematical Basis of Virtual Microscopy

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Additional information is available at the end of the chapter

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1. Introduction

Tissue investigation remains one of the most reliable diagnostic ways in both general medical practice and liver pathology. At present, the routine liver biopsy investigation should include obtaining a representative tissue sample, adequate technological processing and application of histochemical stain panel [1-5]. The evaluation must be done in accordance with up-to-date disease classifications and validated diagnostic criteria [6]. Protocol approach is recommended in order to decrease the variability in description. In case of chronic inflammatory liver disease, semiquantitative evaluation of inflammatory activity by Knodell, Ishak, METAVIR or Scheuer score, or analogous system [7-11] must be applied. Additional methods as immunohistochemistry or polymerase chain reaction are applied by necessity. The morphological evaluation of biopsy is a part of medical teamwork. It should be preceded by clinical and laboratory investigations and biopsy findings must be incorporated in the general patient's information. Many of these principles will remain in use in the nearest future. However, both clinical diagnostics and medical research undergo almost unlimited progress. The upcoming innovations in liver biopsy analysis include incorporation of digital image analysis, genetic investigations and immunohistochemistry for functionally important molecules as cytokines, cell cycle markers and viral life cycle markers into everyday practice.

2. Morphological evaluation of liver: Today's reality

Despite the fact that the histological assessment of liver tissues plays an essential role in the diagnosis of liver diseases and the histological conclusion serves quite often as a basis for

establishing the diagnosis, there are factors or reasons to be taken into consideration which can negatively influence the results obtained in morphological evaluation of liver biopsy.

We have "assessed" the percentage of each factor's influence on the final result– description and conclusion regarding diagnosis, where "0" is considered a factor that does not affect the evaluation and its outcome, but "100%" – the factor which actually hinders the correct diagnosis of the disease. The factors that may affect the liver tissue morphological assessment and diagnosis of disease are summarized in Table 1.

No.	Factors affecting liver tissue morphologic assessment	Evaluation of impact on final outcome in % *	Comments
1.	Biopsy site selection in liver tissues	100 – 0	It can be completely non-representative site, such as subcapsular
2.	Fixation and transfer of biopsy sample	100 – 0	Chemical environment (composition) and temperature of fixation can affect the biopsy specimen
3.	Selection of certain section out of the whole sample	50 – 0	If the whole sample is used, then the error theoretically is not possible
4.	Selection and quality of staining (panel of visualization methods) of the sample	100 – 0	If the sample is not stained for the reason to label a certain substance, e.g., Fe, the error can reach 100%
5.	Quality of biopsy specimen sections or microtomy	30 – 0	Thick or disrupted tissue sections can hinder the pathology from the observer. The thickness should not exceed 3-4 micrometres.
6.	Number of viewable visual fields	80 – 0	Inaccuracies can occur if only some separate visual fields are examined
7.	Technical condition of the microscope	80 – 0	Incomplete quality of optical system can hinder the pathology from the observer.
8.	Selection of evaluation scale	100 – 0	If the specimen where the basic pathology relates to fatty changes (steatosis) is assessed according to Knodell scale, then the assessment is inadequate if compared to the actual liver tissue damage

* 100% - affecting

0% - not affecting

Table 1. Factors affecting liver tissue morphologic assessment

http://dx.doi.org/10.5772/52753

Further we will have a look at each factor separately. The first reason which may significantly affect the final result is the incidental character of biopsy specimen collection by means of "blind" biopsy. In case of diffuse liver damage, it is important to obtain liver specimen from a representative site (which is not subcapsular) or under ultrasonographic (USG) control. If the liver specimen is obtained during invasive procedure (laparoscopy or open abdominal surgery), it is of high importance to give information about preferable biopsy site to the colleague obtaining liver specimen. The liver specimens obtained during surgery are certainly more targeted. More or less qualitative methodological performance of tissue collection may also cause certain imperfections affecting quality of specimen evaluation.

Presuming that the biopsy specimen is obtained from the site typical for the certain liver pathology, one more important issue is the quality of biopsy specimens' fixation and slicing.

The aspect of „special" tissue staining must also be looked at, because in case of absence of examination request or list of preliminary diagnosis provided by clinician, that emphasizes the need for particular staining, morphologist is unable to give an adequate diagnostic assessment of biopsy specimen. Thus diagnosis like haemochromatosis and other pathologies known as "storage diseases" can be missed.

The next factor, i.e., selection of certain section out of the whole biopsy specimen, is an issue arising only in case if the biopsy specimen is not examined throughout or along its horizontal length. The cross-sectioning gives the chance to analyze tissues on different "depths" or „levels" of the biopsy specimen.

The technical condition or quality of the microscope and number of viewable visual fields are to be considered seriously. Nowadays, the usual practice of the pathologist is a general overview of the material to gain insight into overall picture, noticing the most typical and important peculiarities. Inaccuracies can occur if only some separate visual fields are examined.

The subjective component of the morphological assessment of liver specimens and interpretation of the observed changes and their compliance or adherence to one or the other pathology is essential also. The problem could be the qualification and experience of clinician to put together or combine visual insight in the particular biopsy specimen and clinical diagnosis made up of biochemical, immunological and genetic parameters, and to use the interpretation of morphologist properly for establishing the diagnosis.

Selection of morphological or histological evaluation scale is significant. These scales are very advantageous for standardizing expert's assessment, converting it into measurable characteristics and helping the clinician to make final decision about patient's diagnosis. In case of light microscopy the issue of selection of evaluation scale is a factor with up to 100% error probability. For example, the use of the Knodell scale for patient with steatohepatosis, HAI = 0, leads to incorrect conclusion that the patient is healthy, especially if the biochemical parameters of blood are not altered.

If in addition the electron microscopic investigation of sequential liver biopsy specimens are done, obtained results and conclusions are also affected by the whole process of the above mentioned biopsy specimen collection and processing. The electron microscopy is currently consid-

ered as an auxiliary method or technique, yet in the age of high-tech medicine, processes ongoing on the level of organelles are the ones which by characteristic ultrastructural changes frequently refer to or indicate a particular pathology. The following must be strictly observed in electron microscopy: 1) liver tissue sampling and slicing into 1 mm^3 pieces without mechanically squeezing them and immediate immersion in fixing solution; 2) chemical composition of fixing solution, temperature, sample fixation and rinsing time; 3) embedding of liver tissue samples in mixture of epoxy resins in accordance with polymerization time of these resins; 4) quality of sample cutting with ultramicrotome and contrasting with uranyl acetate and lead citrate; 5) all cells and their organelles visible in the ultra-thin slices under the electron microscope have to be examined. It should be noted that resolution of transmission electron microscope (TEM) is within the range of 0.2 to 2 nm and resolution of scanning electron microscope is 4 nm.

3. Virtual microscopy: The general principles

To reduce the potential inaccuracies in the processing and evaluation of biopsy specimens it is important to look for modern solutions in order to maximize the efficiency of use of biopsy specimens. One of the solutions could be application of virtual microscopy having extensive mathematical basis with fractal and entropy considerations as well as technological support by appropriate software and hardware. Implementation of innovations into practice could significantly increase the effectiveness of liver biopsy specimens.

The digital image analysis [12-14] and computed morphometry in general is considered an important tool in pathology. It can decrease the workload of voluminous repeated measurements and increase the accuracy and objectiveness of the results. In several fields, e.g., immunohistochemical and molecular typing of breast cancer, the application of digital image analysis is already highly practical [13].

In virtual microscopy, the demands for mathematical basis are higher than in routine histology. This is illustrated by examples of entropy considerations, Delaunay's triangulation or fractal geometry and general non-Euclidean geometry for irregularly shaped biological objects [14-16]. Sophisticated software must be elaborated as well. Additional technical requirements exist for image resolution and size, fast wide-band data transfer as well as digital data storing [12, 13]. The slide scanners and visualisation software are available and improve continuously [12].

Computed morphometry becomes more practical in association with virtual microscopy and digital image analysis as well. As postulated in reference [16] the natural development of science occurs from the ability to recognize, name and classify the object (corresponding to the diagnosis, e.g., chronic viral hepatitis C) to semiquantitative, ordering measurements (e.g., the activity assessment by Knodell or any analogous scale), finally reaching quantitative characteristics. Descriptive diagnoses and semiquantitative estimates are widely used in the „classic" liver pathology. In order to gain sufficient reliability and fastness, scalar measurements would require digital assessment [16]. Computed morphometry on the basis of virtual microscopy is a way towards scalar measurements.

http://dx.doi.org/10.5772/52753

The virtual microscopy can be performed in two different ways. Interactive virtual microscopy by whole slide imaging leaves the conclusion in the hands of pathologist. It changes significantly the working tools from optical microscopy and subjective decisions to computer screen and objective measurements. The automated virtual microscopy is even more exciting as computer system should evaluate the diagnoses [14].

In liver pathology, the software develops regarding assessment of steatosis [17-19] and fibrosis [20-24]. Necroinflammatory changes can be quantified as well [16].

Regarding liver ultrastructure, morphometric evaluation of hepatocyte volume can have prognostic significance predicting survival as shown in liver cirrhosis associated with portal hypertension [25]. Morphometric analysis of liver parenchyma in different alcohol-related pathologic conditions has been tested with good results [26]. Thus, changes in the volume fraction of parenchymal interstitial space and in the surface density of hepatocyte plasma membrane, rough endoplasmic reticulum and outer mitochondrial membrane can be of importance for distinguishing between cirrhosis and non-cirrhotic states. Hepatocyte nuclear volume fraction measurement can predict the survival in case of cirrhosis. Interestingly, few images are necessary to perform these measurements thus helping to characterise even scarce tissue material [26].

Combination of multiplex quantum dot immunostaining with high resolution whole-slide digital imaging and automated image analysis has been described [27].

At present, the two most frequently studied targets for computer-assisted and/or digital image analysis in liver biopsies include steatosis and fibrosis.

4. Digital assessment of liver steatosis

Among Western population, liver steatosis is a frequent finding [28-29] as it is associated with such common factors as chronic viral hepatitis [19], alcohol drinking, diabetes mellitus or obesity [17]. It has been considered a risk factor for liver fibrosis [18, 19]. Steatosis, including non-alcoholic steatohepatitis [19] has become an important target in diagnostics and scientific research therefore highly reproducible measurements are necessary to evaluate the course of disease, outcome and effect of treatment. The biopsy is still considered a gold standard in the diagnosis and assessment of steatosis as the imaging including ultrasonography, computed tomography and magnetic resonance imaging can be affected by lower sensitivity [17, 30]. The severity of steatosis in liver biopsies can be graded by several semiquantitative systems (Table 2) assessing the eyeballed proportion of affected cells [30-35].

The present semiquantitative estimates are subjective and limit the possibilities of statistic analysis [18]. Numerical value, expressing the exact percentage of affected cells would be more reliable if an adequate biopsy is analysed. Such measurement is possible, especially in computer-assisted way, but it would require architecturally arranged count of nuclei and fat vacuoles per biopsy. Thus, the measurement would be time-consuming and accordingly expensive. On the other hand, steatosis is relatively easy target for digital quantification of the

general fat amount due to the regular shape and distinct colour of fat vacuoles [18, 19]. The digital quantification of steatosis shows high reproducibility exceeding the quality of manual estimate [19]. Commercial software for image analysis has been recently employed and novel automated procedures are under development [18]. The estimate is more reliable if both morphological and chromatic operators are used in order to characterise lipid particles [18]. The fat vacuole is optically and geometrically simple object – optically empty after routine processing and deparaffinisation, thus white and rounded. If colour only is used for identification, however, the sinusoids, empty portal vessels and bile ducts [30] as well as glycogen nuclei in hepatocytes might be undertaken as false positives (Figure 1). The rounded shape of fat vacuole helps to exclude longitudinal or tangential sections of sinusoids, blood vessels and portal bile ducts. In haematoxylin-eosin stained sections, the colour contrast can be used to identify glycogen nuclei as in this case the optically empty space is surrounded by basophilic nuclear membrane in contrast to fat vacuole located in eosinophilic cytoplasm. Thus, the conclusion at present is to include both chromatic, size and shape assessment [18, 30]. Manual check can improve the accuracy in case of perpendicular sections of small vessels and fat cysts [30]. However, such control would increase the workload. The benefits of objectiveness and numerical value of continuous variable still remain. More studies would be necessary to determine how accurate the control must be for practical means; theoretically the significant vascular changes in cirrhosis point towards the idea that accurate identification of fat vacuoles is a must to avoid non-random errors.

Grading	Reference
Mild: less than 30% hepatocytes involved Moderate: 30-60% hepatocytes involved Severe: more than 60% hepatocytes involved	[31]
Grade 0 (no or minimal steatosis): less than 5% hepatocytes involved Grade 1: at least 5% but less than 25% hepatocytes involved Grade 2: at least 25% but less than 50% hepatocytes involved Grade 3: at least 50% but less than 75% hepatocytes involved Grade 4: at least 75% hepatocytes involved	[32]
Estimating the percentage of affected hepatocytes in 5% bands	[30]
Grade 0: less than 1% Grade 1: at least 1% but less than 6% hepatocytes involved Grade 2: at least 6% but less than 34% hepatocytes involved Grade 3: at least 34% but less than 67% hepatocytes involved Grade 4: at least 67% hepatocytes involved	[34] [35]
Grade 1: less than 33% Grade 2: at least 33% but less than 66% hepatocytes involved Grade 3: more than 66% hepatocytes involved	[33]

Table 2. The different grading systems of liver steatosis

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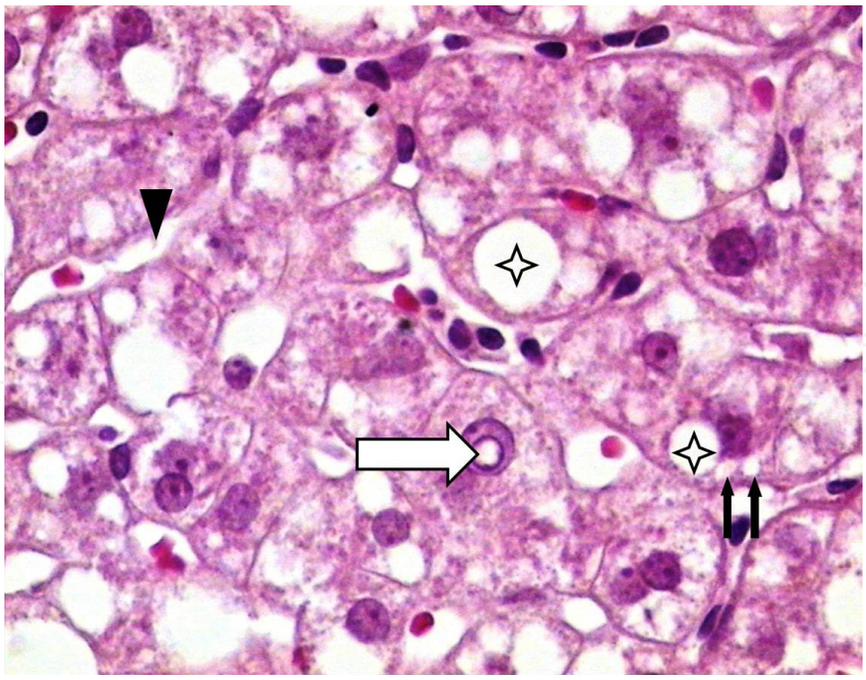

Figure 1. Liver steatosis. Note the macrovesicular steatosis (stars) characterised by size of fat vacuole exceeding the diameter of hepatocyte nucleus, and the microvesicular steatosis (small arrows) caused by fat vacuoles smaller than hepatocyte nucleus. The optically empty fat vacuoles must be promptly distinguished from glycogen nuclei (large arrow) and sinusoids (arrowhead). Haematoxylin-eosin stain, original magnification 400x

The fat stains as Sudan IV are well-known [4]. However, several researchers have reported technical problems. The artifacts can include deformation of lipid vacuoles as well as sinusoidal and background staining [17, 36-38]. The non-lipid positivity would limit the possibilities of colour analysis, and the deformation – of shape analysis. The practicality of osmium tetroxide stain is negatively affected by the necessity to use frozen tissue and by the toxicity of reagents [4].

Several research groups have reported that manual assessment of steatosis leads to significantly higher estimates than computer-obtained data [17, 19] regardless if area measurement or stereological point counting is used [30]. The coefficient can be as high as 3.78 [19]. Practicising physicians should remember that association between degree of steatosis and risk of cirrhosis is proved using manual assessments and thus the scales are adjusted for manual use. Consequently, interpretation of digital data cannot involve the use of unadjusted previous scales as risk classes.

It should be noted that the principal meaning of diagnosing steatosis is not affected by the evaluation method. Increasing steatosis percent is associated with advancing fibrosis stage both manually and digitally [19]. The data obtained by pathologist and automated software show close correlation [17]. After liver transplantation, aspartate aminotransferase, alanine aminotransferase and prothrombin time have shown better correlation with automated measurements in 4 of 5 posttransplant time points but the total bilirubin level correlated better with manual assessment in 3 of 5 time points. The graft survival showed a significant association with macrovesicular steatosis both in automated and manual measurements although the p value was less for automated measurement [17].

When analysing liver steatosis, the observations of higher accuracy in resin-embedded samples [18] request more technological progress in order to create methodology for easy use in routine samples.

Digital stereological point counting has been employed in liver steatosis evaluation as well [33]. The researchers have observed the same fact that manual semiquantitative assessment tends to be significantly higher. The lack of precision in manual evaluation can be related to the physiology of vision and processing of the visual information [19, 39].

Some researchers have also come to the conclusion that automated assessment of liver steatosis is more time-consuming than manual [30]. The time input for digital measurement is found to be threefold greater than for manual evaluation [19]. Although this opinion is based on trustable experience, half of the problem is solved already as the whole slide imaging eliminates the need to choose appropriate number of representative fields submitted for analysis and the necessity for human participation in the obtaining and archiving of digital images. Besides the whole slide imaging, the degree of automatisation must be further increased: optimal software abolishes the manual correction of object inclusion into measurements. However, this deserves morphologically correct mathematical model. Other groups have considered computer-aided morphometry to be fast and objective [16].

5. Digital assessment of inflammation in liver biopsy

The computer-aided assessment of necroinflammatory processes in chronic viral hepatitis has been tested. To perform this, immunohistochemical visualisation is necessary in order to highlight inflammatory cells. The application of immunohistochemistry increases the expenses. This drawback can be counterbalanced by gains of rapid measurement, resulting in rigorous results expressed in scalar numbers as well as by complete characteristics more exactly reflecting the status of the whole organ [16].

The assessment of hepatic fibrosis and the closely related architectural deformities as bridging fibrosis and liver cirrhosis have important role in the diagnostics, treatment and prognostic evaluation of chronic liver diseases [24]. The studies of liver fibrosis are facilitated by standard use of special stains for the routine evaluation of liver biopsies in case of diffuse liver disease. Masson's trichrome is an efficient method to highlight fibrosis [3]. The sharp contrast between blue collagen and red parenchyma allows visualisation of even small excess amounts of collagen [23]. Sirius red stain has also been employed [21, 40]; it has the benefit of selective staining of collagen but not proteoglycans [22]. Not surprisingly, comparatively many authors have applied digital image analysis to quantify fibrosis in liver tissue [24]. Validation studies of computer-assisted morphometry have also been performed [21]. Besides the well-developed methodology including software, the application of computer analysis has resulted in exact numerical data allowing detection of interesting biological relationships. For instance, the correlation of fibrosis burden with end-stage liver disease score, serum total bilirubin and international standard ratio of prothrombin has been shown in hepatitis B-related decompensated cirrhosis. Thus, the correlation between the amount of connective tissue in cirrhotic liver and

http://dx.doi.org/10.5772/52753

hepatic functional reserve was demonstrated [24]. The problem was insufficient accuracy of computer-assisted morphometry [21] manifesting as inter-observer differences. Poor correlation of the fibrosis area with Ishak staging score has been observed as well [21]. Other scientists have also found that analysis of early fibrosis necessities qualitative assessment despite the general correlation between amount of connective tissue and Ishak grade of fibrosis [20]. Tissue geometry differences in subsequent sections also can be more accurately classified by human eye [22]. Full section digital analysis seems to be important [20].

Digital image analysis for the evaluation of fibrosis in chronic viral hepatitis C has been studied also as mentioned in references [41-42]. Automatic quantification of liver fibrosis including the validation of the method has been performed as described in reference [43]. Other investigators have employed computerised image analysis for the evaluation of fibrosis as well [44-47]. In most investigations, correlation between digital and manual semiquantitative score has been shown [20, 44-47]. However, the digital data do not allow to differentiate between low stages of fibrosis [20, 45, 47].

6. Digital biopsy analysis for inflammatory liver lesion: Future begins today

The incorporation of Mandelbrot's fractal geometry [48] into the digital evaluation of liver biopsy for chronic hepatitis has brought revolutionary changes [40].

The short description of fractal is provided in Table 3; detailed characteristics can be found in recent reviews [49].

Definition and essential features of fractal	The fractal is a mathematical object characterised by self-similar patterns. At every scale, fractal shows (infinitely) either the same structure or is at least similar to other scales. The complexity is retained independently of magnification. Thus, although fractal curve is one dimensional similarly to regular line, the fractal dimension is greater than topological dimension. Due to the infinite similarity, fractals cannot be measured in traditional ways. Although fractals have got significant popularity due to their beauty, the importance of fractal theory is in the mathematical basis and the ability to describe, among other processes, the biological phenomena.
Fractals in nature: selected examples	Beds of rivers, irregularity of coastline, profiles of mountain chain, clouds
Fractals in biology: selected examples	Branching of blood vessels or bronchi, the invasive edge of tumour, neurons. See also Figure 2-6
Peculiarities of fractals in biology	Biological fractal-like objects have limited range of self-similarity upon magnification thus behaving as random fractals, in contrast to mathematical/geomaterical constructs with unlimited level of complexity (self-exact fractals)

Table 3. The characteristics of fractals

Figure 2. Highly irregular structure of biological object. Use of Mandelbrot's fractal geometry is suggested to describe targets with remarkable degree of complexity and irregularity. Note also the similarity of complex, branching outline with Figures 4 and 5

Figure 3. Retained irregularity of the biological structure at higher magnification: note the remarkable similarity with Figure 2. The persisting complexity at different levels of magnification is another feature suggesting the necessity for fractal analysis. The inflammation in liver biopsy (shown in Figures 4 and 5) depicts analogous features

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Hurst's exponent is another albeit related mathematical construct with major meaning in the digital analysis of liver biopsy. It was first used to study the variation in water flow in Nile basin during the construction of the Aswan dam [16, 50]. In general, it can be used to detect the irregularity – a key parameter analysing the activity of inflammation in the liver as the active inflammation manifests with periportal piecemeal necrosis causing irregularity in the normally smooth border of portal field. Hurst's exponent also can be detected by fractal mathematics. It can describe quantitatively the deviation from smooth contour in natural fractal objects.

To detect the border of inflammatory cell cluster, Delaunay's triangulation can be used with success. In general, Delaunay's triangulation involves set of points in such way that no point is inside the circle drawn through 3 points. It maximizes the minimum angle avoiding narrow triangles. If circle drawn through 2 input points contain the third point in the outside, these points form Delaunay's triangle. The method can be used to mesh the space. By this triangulation, lines were drawn in the scanned image of liver biopsy through each pair of adjacent inflammatory cells resulting in network of triangles showing common border. The most external triangle short sides formed the border of inflammatory cell infiltrate. The triangle side was defined as appropriately short if it was equal of less than 20 microns based on empiric analysis. After the cluster has been outlined, both the amount (by area) of inflammatory cells and the border irregularity and area of cluster-affected tissue can be evaluated [16].

The mathematical basis of so-called geometry of irregularity (Figure 2-3) has allowed to detect the amount of residual liver parenchyma, inflammation (Figure 4-5) and fibrosis (Figure 6-7) as well as to provide index characterising the appropriateness of liver tissue structure (named tectonic index by the authors).

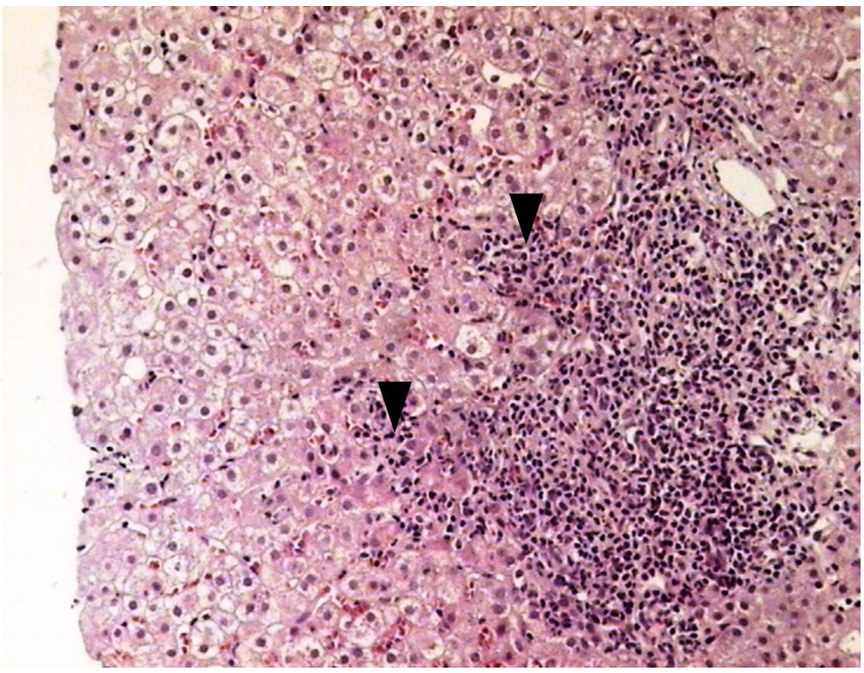

Figure 4. Irregular outline (arrowheads) of portal field in chronic active hepatitis. Haematoxylin-eosin stain, original magnification 100x

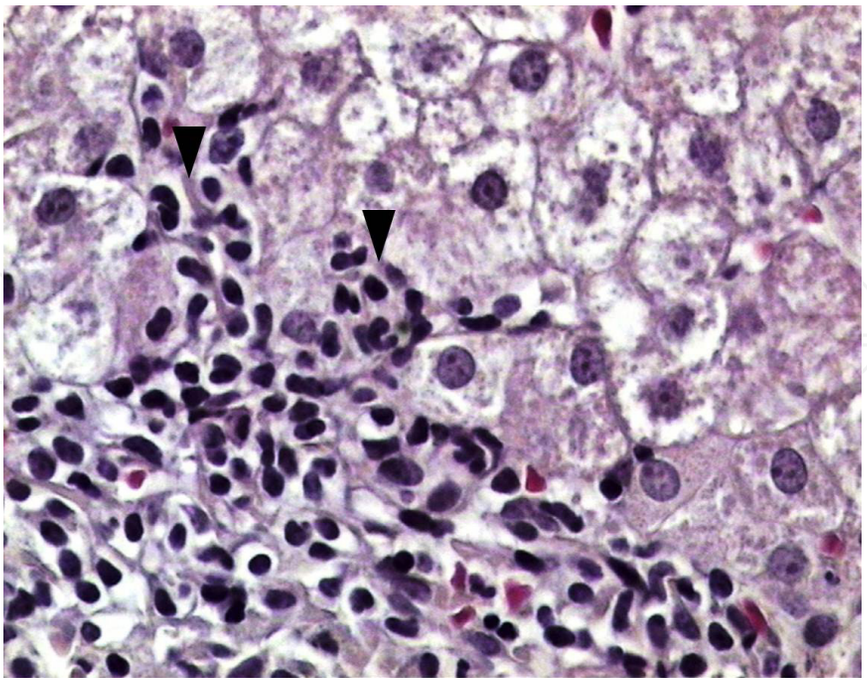

Figure 5. Branching pattern (arrowheads) of periportal inflammatory infiltrate. Note the remarkable similarity with Figure 4 analogous to the relationship between Figures 2-3. The fractal nature of inflammation is thus highlighted. Haematoxylin-eosin stain, original magnification 400x

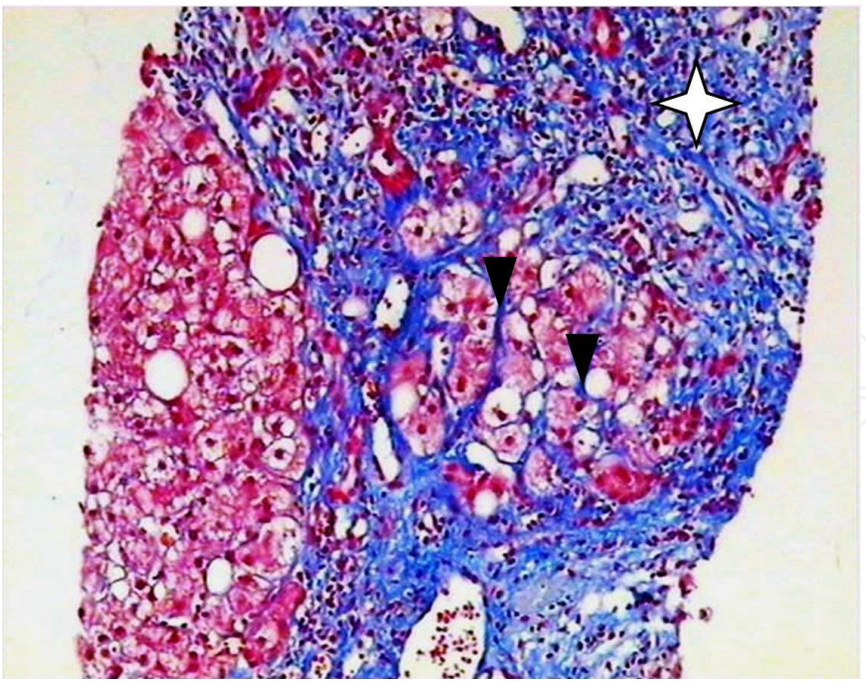

Figure 6. Branching outline of connective tissue fields in liver cirrhosis. Note both the large areas of connective tissue (star) and the thin septa (arrowheads). Masson's trichrome stain, original magnification 100x

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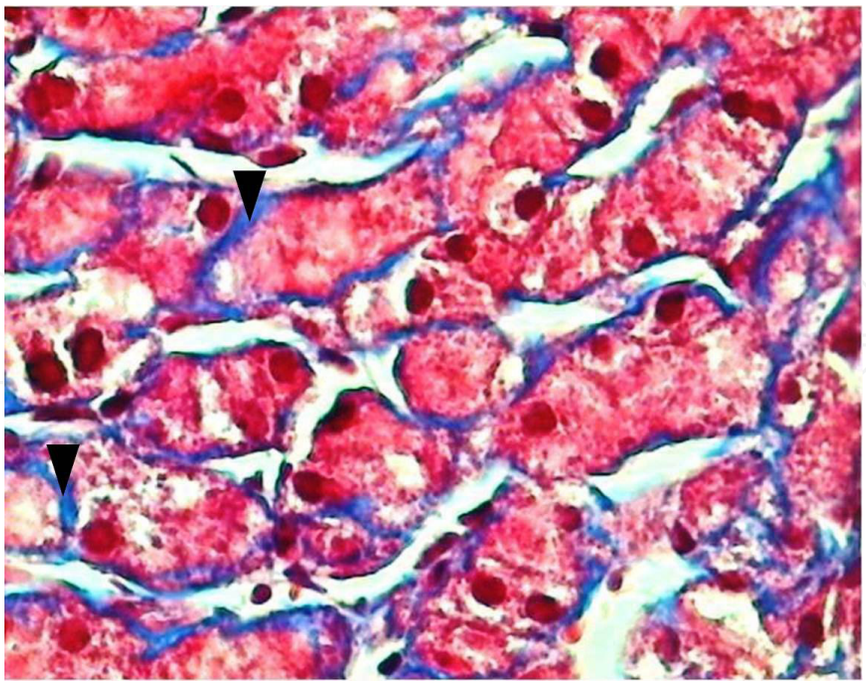

Figure 7. Branching pattern of connective tissue fields in arachnoid liver fibrosis (arrowhead). Masson's trichrome stain, original magnification 400x

The Dioguardi Histological Metriser machine, described in reference [40] is able to produce measurements and even simple diagnoses, working with reasonable speed. The relevant equipment ensures microscope focusing and full slide scanning, and determines the above mentioned parameters excluding any unfilled spaces as vessels, sinusoids, biliary ducts and artifactual holes. The system is able to identify and exclude the Glisson's capsule from the analysis. Colour thresholds are used to select the areas of interest. The inflammatory cells are identified by immunohistochemical visualisation of leukocyte common antigen. For the analysis, the inflammatory cell clusters are outlined by imaginary line connecting the centres of the outermost cells; after that the area of clusters is measured. Thinking in the usual terms, the portal and periportal infiltrates are characterised by this measurement; the portal fibrosis also can influence this measurement providing homing space for inflammatory infiltrate. The area of extra-cluster inflammatory cells is measured separately; these could mostly correspond to intralobular infiltrate. When analysing fibrosis, area of fibrotic tissue is measured. The wrinkledness is detected as the ratio between the perimeter and area of an object. As portal field in healthy liver is smooth, the concept of wrinkledness is an efficient way to detect periportal inflammation and portal fibrosis. The irregularity of collagen islets necessitates the correction by fractal dimension; the fibrotic foci are considered truncated planar fractals. The residual parenchyma is characterised by the tissue area that is not occupied by inflammatory cells and fibrosis. Finally, the loss of order is characterised mathematically. In order to characterise the course of the disease in analogue with the usual staging, the individual fibrosis scalar is compared with the curve of fibrosis development over the course of disease detecting the percentage of the disease course before collagen deposition reaches the maximal

tolerated level of 32% [40] or approximately 36% in liver cirrhosis necessitating liver transplantation [24]. Thus, three approaches are combined: the outlines of regular structures as vacuoles are characterised by traditional, non-fractal geometry, the area of fibrosis and parenchyma are detected using the traditional measurements corrected by the fractal dimension, and the tectonic index is based on the relationships between the Euclidean and fractal dimensions of liver tissue. One of the many positive features of this system is the ability to generate continuous scalar variables. When analysing dynamics in repeated liver biopsies by scalar data, naturally, less biopsies are characterised as lacking significant changes.

Although fractal concept is used in medicine, including at least microscopy, neuroscience and ophthalmology as well as automated measurements not limited to pathology [49, 51, 52], the study described in reference [40] is remarkable as it is highly sophisticated and practical; it is understandable that the research group considers their machine as an intelligent collaborator – and this is exactly the way how future biopsy analysis should proceed.

7. Functional liver tissue analysis in biopsy

The diagnostic evaluation of liver biopsy is mostly based on panel of histochemical stains including hematoxylin-eosin [2], Masson's trichrome [3], PAS [5] and Perl's [1] stains as well as others by necessity. These visualisation techniques should be complemented by various "- omics" tools [27] to gain more data on the function of liver cells. The cytokines, inflammatory mediators, viral proteins, cell cycle proteins and apoptosis markers can be detected; metabolic pathways can be investigated as well. At present, most or proteomic and genetic studies are carried out for scientific research in order to outline the pathogenesis of different diseases. However, in future it could be advisable to include such studies with validated value in the routine investigation as technically the amount of tissue in liver biopsy is sufficient.

Cytokine expression can be analysed, e.g., TGF, EGFR and others [25]. When studying interleukin-6 (IL-6) expression in liver biopsies, higher IL-6 expression was found in non-alcoholic steatohepatitis than in steatosis. Correlation between IL-6 expression and degree of inflammation and stage of fibrosis was detected as well [53]. Due to the complex nature of cytokine action, wide spectrum of different molecules and their receptors must be analysed in details in order to avoid insignificant or contradictory results. This leads to a clear-cut necessity for virtual microscopy and digital image analysis. Toll-like receptor-4 (TLR4) expression can be analysed in liver biopsy by immunohistochemistry. The expression of TLR4 has been shown in hepatic progenitor cells and interlobular bile duct epithelium in correlation with stage of liver disease, grade of liver inflammation and activity of portal/septal myofibroblasts [54]. The expression of interferon stimulated gene 15 can be analysed by IHC at protein level; up-regulation in hepatocytes is more pronounced in patients not responding to interferon / ribavirin treatment in contrast to predominant expression in Kuppfer cells in treatment responders [55]. Proteomic studies including immunohistochemistry in liver biop-

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sy have targeted cell structure-associated proteins - actin, tropomyosin, transgelin and human microfibril-associated protein 4 in order to identify biomarkers of liver cirrhosis [56]. COX-2 is over-expressed in chronic hepatitis C and the expression decreases following treatment with interferon alpha regardless of sustained virological response [57]. Increased endoglin and TGF beta 1 expression is significantly associated with progressive hepatic fibrosis in chronic viral hepatitis C [58].

Cell cycle analysis can add valuable information [59]; digital image analysis should be added in the logistics again. Arrested cell cycle status has been demonstrated in chronic hepatitis C infection analysing the expression of mini-chromosome maintenance protein-2 as higher sensitivity proliferation marker, G1 phase marker cyclin D1, S phase marker cyclin A, cell cycle regulators p21 and p53, apoptotic protein caspase 3 and anti-apoptotic protein Bcl-2 [60, 61]. When analysing liver biopsies from patients with chronic viral hepatitis C, higher G1 and lower S phase fractions has been found also by Werling *et al*., employing image analysis method [59]. Apoptosis-related pathways can be explorated including evaluation of Bax, Bcl-xL and Bcl-2 proteins [62]. Thus, hepatitis C virus infection can deregulate the cellular processes [63] and it can be practical to reveal the way and degree of the regulatory shift.

Viral antigens including hepatitis C antigen can be detected in liver tissue by immunohistochemistry [64]; the finding can be helpful in cases with difficult differential diagnosis or combined liver pathology. The association of expression pattern with fibrosis may suggest pathogenetically important information as well [64].

Metabolic pathways can be evaluated in liver biopsy. For instance, widespread expression of vitamin D receptor has been shown in the hepatocytes and inflammatory cells in case of chronic liver disease including non-alcoholic steatohepatitis and chronic viral hepatitis C. The expression decreases as the liver histology is damaged [65].

Inflammatory cells are as important components in diffuse liver disease as the hepatocytes. Thus, higher numbers of intrahepatic follicular T-helper lymphocytes in conjunction with IL28B polymorphism analysis is found to be strongly predictive of treatment response using pegylated interferon and ribavirin [66]. CD4+ regulatory T cells can be evaluated [67].

Logistic structures have been implemented to develop next generation toolkits for automated image analysis to enable quantification of molecular markers. The group of researchers [27] have collaborated within open source image analysis project [68] to reach effective output by combination of quantitative analysis, multiplex quantum dot (nanoparticle) staining and high resolution whole slide imaging to detect nine different fluorescent signals for multiple antigens [27].

DNA microarray technology has enabled genome-wide analysis of gene transcript levels. This technology has been applied in order to compare gene expression profiles at different stages of chronic hepatitis C and hepatocellular carcinoma in the setting of hepatitis virus C infection [63, 69]. Hundreds of genes involved in carcinogenesis, cell growth, proliferation and death are differently expressed in advanced viral hepatitis C in comparison to early viral hepatitis C or non-viral hepatitis [63]. In chronic hepatitis C, the up-regulation involves

genes related to metabolism and immune responses. In hepatocellular carcinoma arising in hepatitis C patients, genes associated with cell cycle, growth, proliferation and apoptosis are up-regulated [69]. Chronic hepatitis B and autoimmune liver disease have been studied by this technology as well [70]. In advanced chronic viral hepatitis B, genes associated with extracellular matrix turnover, cell growth and DNA repair are up-regulated but the expression of genes regulating complement activation and innate immune response is decreased. In early disease stages, the gene expression is different in case of chronic viral hepatitis B, autoimmune hepatitis and primary biliary cirrhosis. Chronic viral hepatitis B is associated with expression of genes considering chemotaxis and cell homeostasis; autoimmune hepatitis – with down-regulation of genes associated with protein binding, but primary biliary cirrhosis in early stages involves the actin and myosin gene expression. As chronic viral hepatitis B progresses, the expression of genes regarding signalling pathway, cell communication, collagen turnover, chemokine ligands and metallothionein changes [70]. The findings are of major interest displaying the pathogenesis of different inflammatory liver diseases and neoplastic transformation. Diagnostic consequences should follow soon as the differential diagnosis of inflammatory liver diseases regarding aetiology can represent a difficult task.

The level of mRNA can be post-transciptionally regulated by micro RNA (miRNA). The regulation of biological processes by miRNA is shown also in case of such canonical diffuse liver disease as chronic viral hepatitis C. Technological studies have been conducted using biopsy material [71]. Transcriptome analysis has shown prognostic value, e.g., in order to predict the severity of fibrosis progression after liver transplantation in recurrent viral hepatitis C patients [72].

8. Conclusions

Liver biopsy investigation could soon shift from routine light microscopy to digital image analysis by virtual microscopy and incorporation of numerical measurements in conjunction with integrated analysis of cell functions at DNA, RNA, protein and signalling level. This shift could lead from static to dynamic tissue evaluation. The technological logistics should include the best standards of tissue fixation, processing, microtomy and visualisation complemented by automated immunostaining, full slide scanning to ensure complete digital analysis and optimal choice of software considering the biological appropriateness of the analysis algorithm.

As the diagnostic electron microscopy is continually developing, we expect that in future it will be used in hepatology as an auxiliary method, based on digital analysis of electronograms. Liver biopsy analysis using transmission and scanning electron microscope could continue to provide important additional information in diagnostic hepatology and scientific research of liver diseases, as well as it could help to study unresolved molecular mechanisms regulating liver cells' functions. In future the ultrastructural studies of liver biopsy in hepatology will probably be associated with assessment of liver tissues in cases of liver transplantation, with studies of new medicinal products – detection or exclusion of their po-

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tential hepatotoxic effect, with identification of viruses, as well as with determination of influence of various environmental hazards.

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