Histomorphometric Findings May Help Predicting Response in Patients with Chronic Liver Disease

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ABSTRACT **Background:** Hepatitis C virus (HCV) is a leading cause of cirrhosis and hepatocellular carcinoma worldwide. Several viral and host factors related to viral response have been reported in the era of treatment with pegylated (PEG)-interferon and ribavirin.

Objectives: To quantify histological findings from patients with chronic HCV using computerized morphometry and to investigate whether the results can predict response to medical treatment with peg-interferon and ribavirin.

Methods: We followed 58 patients with chronic HCV infection with METAVIR score F1 and F2 in our liver unit who were grouped according to treatment response sustained viral response (SVR) and non-SVR. Liver needle biopsies from these patients were evaluated and histological variables, such as inflammatory cells, collagen fibers and liver architecture, were quantified using computerized morphometrics. The pathologist who performed the histomorphometric analysis was blinded to previous patient clinical and histological information.

Results: Histomorphometric variables including the density of collagen fibers were collected. The number of inflammatory cells in the portal space and textural variable were found to be statistically significant and could be used together in a formula to predict response to treatment, with a sensitivity of 93% and a 100% specificity.

Conclusions: Histomorphometry may help to predict a patient's response to treatment at an early stage.

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KEY WORDS: hepatitis C virus (HCV), interferon, morphometry, ribavirin, sustained virologic response (SVR)

Over 180 million people worldwide have chronic hepatitis C virus (HCV) infection. HCV is the leading cause of cirrhosis and hepatocellular carcinoma (HCC) worldwide. It is the leading indication for liver transplantation in the United States [1]. Traditionally, treatment of HCV was based on pegylated interferon-alpha (PEG-IFN) combined with ribavirin given for 24 weeks in genotypes 2 and 3 or for 48 weeks in genotype 1 [2]. In recent years, the development of highly potent direct-acting antiviral agents (DAAs), has revolutionized the treatment of HCV with efficacy of > 90% in all genotypes [3], and substantially fewer side effects compared with the classic interferon-based regimens.

Sustained virologic response (SVR) is defined as an immeasurable serum level of HCV RNA 24 weeks after the conclusion of treatment and is the desired outcome of the treatment since it almost always represents cure. Furthermore, achieving SVR prevents liver disease progression [3]. Viral response to treatment with PEG-INF and ribavirin is influenced by both patient (ethnicity, gender, age, liver fibrosis) [4] and virus-related factors such as genotype and pre-treatment viral RNA levels [5-7].

MORPHOMETRY

Morphometry is a discipline exploring the differences in shape, size, and orientation of objects in a tissue. There are several variables for typifying the morphological parameters of a specific object including length, angles, orientation, and spatial distribution.

Morphometry allows the quantification of these variables, making it possible to emphasize areas with significant differences. In recent years, morphometry was used to predict lymph node involvement and disease progression in carcinoma of the vulva and kidney [8,9], dysplasia, progress to adenocarcinoma in patients with Barret's esophagus [10,11], and prediction of the clinical phenotype in Crohn's disease [12].

In liver diseases, morphometry has been used to quantify liver fibrosis following transplant for HCV cirrhosis to predict clinical outcomes post-transplantation [13]. In another study, quantifiable liver fibrosis correlated with the amount of pressure differentials of the hepatic veins (HVPG) [14]. Finally, morphometry was shown to be a valuable tool for following the progress of liver fibrosis in patients with chronic HCV [15].

We hypothesized that by quantifying histological variables, such as inflammation and fibrosis, early in the course of chronic HCV, we may be able to predict the response to anti-viral treatment.

PATIENTS AND METHODS

Study design and population

A retrospective study of patients with chronic HCV was conducted. Patient records were collected from the computerized database of Rambam Health Care Campus, Haifa, Israel. We included patients with chronic HCV (genotype 1) who were naïve to treatment, had a viral load of > 400,000 IU/ml and had a liver biopsy within a year prior to treatment showing F1 or F2 fibrosis according to the METAVIR score [Table 1]. All patients were treated with PEG-INF and ribavirin. Exclusion criteria included patients younger than 18 years of age or older than 65 years of age, experienced patients (patients given anti-viral treatment in the past), patients who stopped the antiviral treatment due to side effects, patients whose liver biopsy was performed over a year before treatment, fibrosis level according to METAVIR score below F1 or above F2, viremia level below 400,000 IU/ml, HCV genotype other than 1, patients with a background of another liver disease, alcoholic patients, or patients with HBV or HIV.

We compared patients who responded to treatment and reached SVR (n=30) to patients who did not respond to treatment (non-SVR) (n=30). Data collection included patient clinical and laboratory data and treatment type and duration.

HISTOMORPHOMETRIC ANALYSIS

The original paraffin blocks were used and new slides were prepared and stained for hematoxylin and eosin (H&E), reticulin, and Masson's trichrome stained. The slides were then scanned by dotslide virtual microcopy Olympus, Japan). Each slide was scanned manually; 3 to 4 representative images were chosen from each slide. The average number of portal spaces in each biopsy was 6–8. The number of fields scanned and the microscope magnification level were identical in each case. For computerized analysis we used the Image-Pro® Plus Version 7.0 (Media Cybernetics, Inc., USA) software and the MATLAB (Mathworks, USA) software. The histomorphometric analysis was blinded to the patient's identity and clinical data.

The histomorphometric variables tested in this study included the number of inflammatory cells in the hepatic portal space, the amount of fibrosis in the hepatic portal space, and parenchymal architecture. [Figure 1].

STATISTICAL METHODS

Data were tested by the Kolmogorov Smirnov test for data distribution. Correlation between variables was determined by Pearson's Chi-square test for continuous variables and Spearman's test for categorical variables. Relations among binary variables were determined using the Chi-square test.

The prediction level was determined by using discrimination analysis. This statistical method tests the relation between continuous independent variables and categorical variables. In

Table 1. Demographic and clinical characteristics

Parameter	Group 1 SVR (n=29) 95% confidence interval or mean ± SD	Group 2 non-SVR (n=29) 95% confidence interval or mean ± SD
Gender		
Male	60%	53%
Female	40%	47%
Age (year)	42 ± 11	47 ± 8.9
BMI kg/m^2	25 ± 3.38	26 ± 3.7
Country of origin		
Ukraine	20%	16%
Russia	67%	70%
Israel	7%	7%
Romania	7%	0%
Kazakhstan	0%	7%
Habits		
Alcohol drinking	50%	13%
Smoking	43%	40%
Laboratory data		
ALT (UNL=60 U/L)	75.3 ± 61	71± 33
ALK. PHOS. (UNL=120 U/L)	73 ± 18	66.7 ± 24
Albumin (LNL=3.2 gr/dl)	4.38 ± 0.46	4.27 ± 0.3
Bilirubin (UNL=1.2 mg/dl)	0.73 ± 0.25	0.68 ± 0.23
WBC (LNL=4000/µl)	6968 ± 1912	5790 ± 1693
Hemoglobin (LNL=11.5 g/dl)	14.6 ± 1.49	13.6 ± 1.49
INR (UNL=1.1)	1.07 ± 0.18	0.98 ± 0.05
Platelets (LNL=150000/µl)	221655 ± 57000	213439 ± 61000
Genotype		
1a	20%	0%
1b	80%	100%
Viral load (before treatment) IU/ml	2887520	3874280
METAVIR fibrosis score		
F1	67%	53%
F2	27%	30%
F1-2	6%	17%
Inflammation		
A1	20%	20%
A2	44%	36%
A3	6%	6%
A1-2	20%	14%
A2-3	10%	24%
Treatment		
COPEGUS+ PEGASYS 24 weeks	3%	12%
COPEGUS+ PEGASYS 48 weeks	70%	46%
COPEGUS+ PEGASYS 72 weeks	10%	3%
PEG-IFN + ribavirin 24 weeks	3%	3%
PEG-IFN + ribavirin 48 weeks	14%	23%
PEG-IFN + ribavirin 72 weeks	0%	3%

ALT = alanine aminotransferase, ALK. PHOS. = alkaline phosphatase, BMI = body mass index, HCV = hepatitis C virus, INR = international normalized ratio, LNL = lower normal limit, PEG-IFN = pegylated interferon, SVR = sustained virologic response, UNL = upper normal limit, WBC = white blood cells

Figure 1. Quantification of inflammatory cells in the hepatic portal space **[A]** Image of hepatic portal space magnified ×10 scanned in light microscope with TRICHROME staining. **[B]** Red marking of inflammatory cells within the hepatic portal space (border in green). Fibrosis measurement in the hepatic portal space compared to the area: image of hepatic portal space magnified ×10 scanned in light microscope. **[C]** collagen fibers in the liver tissue are stained with TRICHROME staining and appear in blue. **[D]** the hepatic portal space border is shown in green and the collagen fibers in red

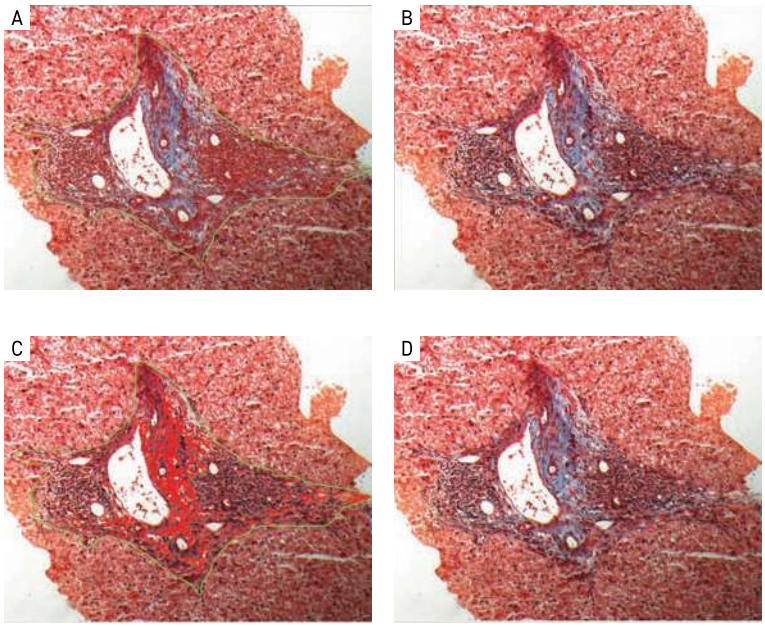

Table 2. Correlation between demographic, histomorphometric and laboratory factors and the response to anti-viral treatment

Parameter	*P* value
Gender	
Male	0.635
Female	0.225
Age (year)	0.05
BMI kg/m^2	0.63
Laboratory data	
ALT (UNL=60 U/L)	0.7
ALK. PHOS. (UNL=120 U/L)	0.1
Albumin (LNL=3.2 gr/dl)	0.1
Bilirubin (UNL = 1.2 mg/dl)	0.7
White blood count (LNL = 4000/µl)	0.026
Hemoglobin (LNL = 11.5 g/dl)	0.048
INR (UNL = 1.1)	0.7
Platelets count (LNL = 150000/µl)	0.968
Standard deviation of density of collagen fibers in portal space	< 0.001
Maximal density of collagen fibers in portal space	0.04
Inflammation parameter	
Absolute number of inflammation cells in portal space	0.05
Portal space area	0.14
Number of inflammation cells/mm^2	<0.001
Architectural parameters	
ENTROPY	0.04
CONTRAST	0.02
HOMOGENIETY	0.04
CORRELATION	0.15
Architectural parameters (MatLab analysis)	
LACUNARITY	0.001
SLOPE average	0.15
SLOPE standard deviation	0.11

ALT = alanine aminotransferase, ALK. PHOS. = alkaline phosphatase, BMI = body mass index, HCV = hepatitis C virus, INR = international normalized ratio, LNL = lower normal limit, UNL = upper normal limit

our study, continuous independent variables were demographic, clinical, laboratory, and histomorphometric data. Categorical variables included the two study groups: patients responding to treatment (first group) and patients who did not respond to treatment (second group).

Using this method, it is possible to determine which independent variables are different between the two groups, and based on these variables it is possible to predict the success or failure of treatment. In addition, we used a neural network (NNET) model to discriminate and predict a response to treatment based on non-parametric data. Statistical analyses were performed using Statistical Package for the Social Sciences software version 11 (SPSS Inc., Chicago, IL, USA) and Matlab (Mathworks USA).

We built receiver operating characteristic (ROC) analysis curves to reach the cut-off points showing the best prediction for response to treatment. All statistical tests were two-sided and a *P* value of 5% or less was considered to be statistically significant.

RESULTS

The study population included 60 patients. Two patients were excluded from the study (one patient per group) due to lack of information about the quantitative level of viremia prior to treatment. Alcoholic patients with daily drinking exceeding 20 grams were excluded. Data for alcohol refers to social drinking versus total abstinence.

Thus, each group included 29 patients. Patient demographic and clinical data are shown in Table 1. Most participants in the study were of Russian origin: 67% in the SVR group and 70% in the non-SVR group. Most patients in the study population were HCV genotype 1b patients. In the SVR group, 20% of patients had genotype 1a, while all patients in the non-SVR group had genotype 1b.

Most biopsies of patients in the study population had fibrosis level of F1. In the SVR group, 67% of patients had fibrosis F1, 27% with fibrosis F2, and 6% with fibrosis level F1-2. In the non-SVR patient group, 53% of patients had fibrosis level F1, 30% fibrosis level F2, and 17% with fibrosis level F1-2. In

Table 3. Clinical and histomorphometric variables distinguishing between the two treatment groups

Parameter	*P* value
Demographic and clinical parameters	
Hemoglobin	< 0.001
Fibrosis analysis parameters	
Standard deviation of density of collagen fibers in portal space	< 0.001
Inflammation parameter	
Number of inflammation cells/mm²	< 0.001
Architectural parameters	
Contrast: maximum	< 0.001
Correlation: average	< 0.001
Lacunarity: average	< 0.001

terms of inflammation level, most patients in the study had level A2 inflammation (44% in the SVR group, 36% in the non-SVR group). The average blood leukocyte count was higher in the SVR as compared to the non-SVR group. In the SVR group, 6% of the patients had leukocyte levels below 4000/µl, while 23% of the patients in the non-SVR group had leukopenia.

The correlation between patient demographic and laboratory characteristics and the response to anti-viral treatment is shown in Table 2. The table shows that age, leukocyte count, and hemoglobin levels prior to treatment were the only statistically significant predictors of response to anti-viral treatment.

The correlation between different histomorphometric parameters and the response to medication according to a univariate analysis is shown in Table 2. As shown in the table, multiple histomorphometric parameters including those related to inflammation, fibrosis and architecture were statistically significant predictors of response to therapy.

A second statistical test, using a discriminant analysis was performed to evaluate the correlation between clinical and histomorphometric parameters and response to treatment. The variables found to have the strongest statistical correlation are shown in Table 3 and they include one clinical variable (hemoglobin level) and five Histomorphometric parameters (fibrosis, inflammation, contrast, correlation, and lacunarity)

Regression coefficients provided by the model (B = slope, Constant = intercept) were used to calculate discriminant scores in both groups based on Fisher's linear discriminant functions equation.

The formula included parameters of histophotometric analysis, textural analysis, lacunarity analysis and clinical parameters which could use it to predict response to anti-viral treatment.

EQUATION SPECIFICITY AND SENSITIVITY

We used ROC curves to find the best cutoff points in these DS which will be able to distinguish between response and non-response to treatment. We also calculated the relative specificity and sensitivity for each cutoff point.

A cut-off of 15.7 provided 93% sensitivity and 100% specificity, that is, if the value of the DS equation is above 15.7, this predicts response to anti-viral treatment and reaching SVR in patients with HCV genotype 1 who were treated with a combination of PEG-INF and ribavarin, while a value below 15.7 predicts failure of anti-viral treatment by this combination.

DISCUSSION

In the past, the accepted treatment for HCV included PEG-INF and ribavirin. In recent years, the accepted treatment is DAAs due to their excellent efficacy and safety. This study was initiated in 2011, when PEG-INF and ribavirin were the standard treatment for chronic HCV infection. We sought to study morphometry in patients with chronic liver diseases and HCV is a suitable etiology since it is easy to accurately assess treatment success. Several factors were previously shown to affect treatment success with PEG-INF and ribavirin. These include viral related factors such as genotype and viral load level, in addition to patient related factors such as age, ethnicity, sex, polymorphism of IL28-B, and level of liver fibrosis [16].

This study was comprised of 60 adult HCV patients who received anti-viral treatment combining PEG-INF and ribavarin, and who were under follow-up at a tertiary hospital. The study population was varied in terms of socio-demographic data and clinical and laboratory characteristics. This study examined the predictive power of demographic as well as laboratory and morphometric variables on the response to anti-viral treatment. Clinical parameters are shown to significantly predict response included age, leukocyte level in peripheral blood, and hemoglobin levels. These parameters were found in earlier studies to be important for predicting the response to treatment.

The novel insight in our study was the ability to find morphometric parameters predictive of the chance for response to anti-viral treatment. These factors include collagen fiber density in the hepatic portal space, the amount of inflammatory cells in the hepatic portal space and additional textural parameters of the liver parenchyma. Using the clinical and morphometric parameters we were able to reach a mathematical equation with high predictive value for the treatment's success.

Liver biopsy is considered to be the gold standard of evaluating the severity of inflammation and liver fibrosis in patients with hepatitis C. It is possible to evaluate the severity of the illness based on histological findings, which could be a highly important prognostic measure [17]. Many studies have shown that the higher the liver fibrosis level, the lower the chances of success of anti-viral treatment.

Using the METAVIR method or other methods to evaluate the severity of inflammation and liver fibrosis provides an inaccurate evaluation. There is much variance between pathologists in analyzing the histopathological findings.

In this study, the morphometric test provides a more accurate evaluation of the amount of inflammation and fibrosis in liver

biopsy. It is possible to more accurately quantify the amount of inflammation and fibrosis in the different parts of liver biopsy compared to the existing methods.

To the best of our knowledge, our study is the first of its kind to find a relation between morphometric parameters in liver biopsy and the chances to reach an SVR after treatment. This study found several histomorphometric variables – the standard deviation of collagen fiber density at the hepatic portal space, the amount of inflammation in the hepatic portal space and textural parameters – with statistically significant power for the prediction of treatment success in univariate analysis. In multivariate analysis, only some of these variables were statistically significant predictors.

Mirza et al. [18] showed that a moderate to severe inflammation level in liver biopsy can predict response to treatment in patients with genotype 4 who were treated with PEG-INF and ribavirin for one year, but this finding was only true for patients with bridging fibrosis or cirrhosis.

This finding was explained by the fact that the level of inflammation in the tissue is correlated with the body's immune response against the virus. Interferon alpha is a cytokine with direct anti-viral activity. By itself is an immune-modulator for clearing the HCV virus from the body. The more inflammatory cells in the liver, the better the immune response to the virus and the more immune-modulatory activity by interferon alpha.

We did not find a study that was testing the link between the amount of fibrosis and inflammation of the liver and the chance for response to treatment. In addition, we found no study that was testing the link between textural parameters of the liver and the chance for response to the treatment.

Hui and colleagues [19] showed that the histomorphometric method is a sensitive method for the quantification of liver fibrosis. That group suggested using this method for following-up the response to treatment.

Arima and co-authors [20] showed that the computerized method for quantifying fibrosis is more sensitive to evaluating the reduction of liver fibrosis following anti-viral treatment in HCV patients than the METAVIR score.

Our study provides tools that could be useful both to physicians and to patients to predict the chance of response to PEG-INF and ribavirin prior to treatment.

In accordance with our baseline hypothesis, for the same level of inflammation or fibrosis according to the METAVIR method, there are morphometric differences in regard to inflammation and fibrosis. These differences were related, according to our findings, to the response to anti-viral treatment. Interferon may accelerate the body's immune response in different ways in different patients, and the morphometric test may be able to identify the patients in which the activity of interferon will be maximal.

Our study has several limitations. First, it is a retrospective study. Second, this study was conducted on patients with HCV. Due to the development of DAAs that are highly efficacious, the importance of this method in HCV patients seems to be low. However, the morphometric method might be used in the future in other liver diseases such as chronic hepatitis B and autoimmune hepatitis to test whether there is a connection to prognosis or response to treatment

In addition, recently there has been preference for non-invasive methods for evaluating the severity of liver damage to replace liver biopsies in some of the patients. These methods include fibrotest and fibroscan [21]; thus, for some of the patients we lack an available liver biopsy for performing the morphometric tests.

CONCLUSIONS

To the best of our knowledge, our study is the first ever to test the relation between morphometric parameters and the chance for response to treatment in HCV patients. In addition, by introducing morphometric analysis to the field of hepatology, the contribution of our work may be broader as this method may be used in various liver disease and may prove to be a useful and valuable tool in the practice of liver diseases.

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Capsule

Effects of underfeeding and oral vancomycin on gut microbiome and nutrient absorption in humans

Direct evidence in humans for the impact of the microbiome on nutrient absorption is lacking. **Basolo** and co-authors conducted an extended inpatient study using two interventions that they hypothesized would alter the gut microbiome and nutrient absorption. In each, stool calorie loss, a direct proxy of nutrient absorption, was measured. The first phase was a randomized cross-over dietary intervention in which all participants underwent in random order 3 days of over- and underfeeding. The second was a randomized, double-blind, placebo-controlled pharmacologic intervention using oral vancomycin or matching placebo. Twenty-seven volunteers (17 men and 10 women, age 35.1 ± 7.3 years, body mass index 32.3 ± 8.0 kg/m^2), who were healthy other than having impaired glucose tolerance and obesity, were enrolled and 25 completed the entire trial. The primary endpoints were the effects of dietary and pharmacological intervention on stool calorie loss. The authors hypothesized that stool calories expressed as percentage of caloric intake would increase with underfeeding compared with overfeeding and increase during oral vancomycin treatment. Both primary endpoints were met. Greater stool calorie loss was observed during underfeeding relative to overfeeding and during vancomycin treatment compared with placebo. Key secondary endpoints were to evaluate the changes in gut microbial community structure as evidenced by amplicon sequencing and metagenomics. The authors observed only a modest perturbation of gut microbial community structure with under- versus overfeeding but a more widespread change in community structure with reduced diversity with oral vancomycin. Increase in *Akkermansia muciniphila* was common to both interventions that resulted in greater stool calorie loss. These results indicate that nutrient absorption is sensitive to environmental perturbations and support the translational relevance of preclinical models demonstrating a possible causal role for the gut microbiome in dietary energy harvest.

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Eitan Israeli

Capsule

Priming NK cells for tumor destruction

Some tumors can evade CD8+ T cells, which are used in several cancer immunotherapies, but natural killer (NK) cells provide another option to target such tumors for immune elimination. **Nicolai** and co-authors used several mouse models to investigate how a cyclic dinucleotide (CDN) agonist for an innate immune pathway called STING potentiates the antitumor activity of NK cells. CDN administration induced type I interferons that directly promoted NK cell activation and simultaneously enabled an indirect pathway of activation driven by induction of interleukin-15 signaling in dendritic cells. Amplification of NK-based tumor immunity may offer a valuable adjunct to CD8+ T cell immunotherapy.

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Eitan Israeli