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TEZĂ DE DOCTORAT

EVALUATION OF LIVER FIBROSIS AND STEATOSIS BY ELASTOGRAPHY AND CONTRAST-ENHANCED ULTRASONOGRAPHY

– ABSTRACT –

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GENERAL PART

Chronic liver disease is a persistent and progressive liver disease characterized by liver damage that persists over a long period. The underlying etiologies of chronic liver disease include chronic liver disease associated with viral hepatitis – hepatitis B (HBV), hepatitis C (HCV), alcoholic steatohepatitis and steatohepatitis nonalcoholic, as well as autoimmune and genetic diseases.

It is very important to be able to identify and quantify steatosis and the degree of fibrosis to prevent possible complications and to be able to keep the process under control. This is possible either through liver biopsy puncture, serological tests – through the prediction and diagnostic scores developed over the years or through elastography techniques, much more used in recent years.

Even though liver biopsy is considered the gold standard in the evaluation of liver fibrosis and steatosis, non-invasive methods by elastography have gained a lot of ground. Even liver biopsy cannot be said to be a perfect technique due to inter-observer variability, without even mentioning the technique's invasiveness and complications.

Therefore, this research paper studies two aspects closely related to each other, the need to implement a new diagnostic technique for liver fibrosis, by evaluating liver fibrosis with the help of contrast substance ultrasound (CEUS) and demonstrating the applicability of elastography in the field of hepatology, namely :

1. Screening of risk populations – in this case, the population of patients diagnosed with type 2 diabetes;
2. Diagnosis of liver fibrosis and steatosis and
3. Liver disease monitoring – this includes monitoring during treatment and post-treatment follow-up from the point of view of liver stiffness.

SPECIAL PART

1. GENERAL OBJECTIVES

a. Assessment of the incidence of steatosis and fibrosis and factors associated with these conditions in a large cohort of patients with type 2 diabetes using Vibration Controlled Transient Elastography (VCTE) and controlled attenuation parameter (CAP).

b. Establishing the feasibility and performance of a new 2D-SWE technique (Aplio i800 from Canon Medical Sistem) for the non-invasive assessment of liver fibrosis and steatosis using Vibration Controlled Transient Elastography (VCTE) with the controlled attenuation parameter (CAP) as the reference method.

c. Dynamic assessment of liver stiffness by VCTE in patients with HCV liver cirrhosis, before and immediately after the end of interferon-free treatment.
d. Determining the performance of contrast-enhanced ultrasound and parametric arrival time imaging (CEUS-PAT) in the diagnosis of liver fibrosis in patients with liver cirrhosis.

Considering the general objectives as well as the clinical applications of elastography techniques, the special part has been divided into 4 chapters. In the first chapter, we included a population screening study - the population of patients with type 2 diabetes, studying the frequency of occurrence of liver steatosis and fibrosis on a sample of 776 subjects. This study draws attention to the importance of screening for fibrosis and steatosis in this population through the results demonstrating that 60.3% of subjects had severe steatosis and 19.4% had advanced fibrosis. In the second chapter, the one with the diagnosis of liver fibrosis and steatosis, we included a prospective study, carried out on a cohort of 112 subjects - a part with chronic liver disease and a part healthy subjects, who were used as control subjects. We evaluated the utility in the diagnosis of liver fibrosis and steatosis of a new 2D-SWE elastography technique implemented on the same ultrasonography system, using elastography (VCTE) with CAP as the reference method, thus demonstrating a strong correlation between the methods and very good diagnostic performance. In Chapter 3 we evaluated the utility of elastography in disease monitoring, from diagnosis to post-treatment outcomes in a cohort of 225 subjects diagnosed with hepatitis C virus (HCV). The results were consistent with the literature, with the degree of liver fibrosis decreasing in a significant percentage of subjects. The last chapter focused on contrast-enhanced ultrasound (CEUS) through the PAT module (the time in which the contrast agent reaches the liver, relative to the kidneys, calculated from a colour map) and the usefulness of its introduction in clinical practice. We demonstrated that CEUS-PAT can identify and at the same time exclude the diagnosis of liver cirrhosis, helping in the diagnostic algorithm of focal liver lesions.

2. MATERIAL AND METHODS

A total of 1177 subjects were enrolled in the studies. All subjects gave their written consent to enrol in the study, to be evaluated ultrasonographically, by elastography, biologically and clinically. Each study had well-defined inclusion and exclusion criteria.

Clinical and biological evaluation

Anthropometric, demographic, clinical and biological data were collected on the same day as the elastographic measurements. Biological data included information on

haemoglobin, hematocrit, leukocytes, platelets, cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamyl aminotransferase (GGT), alkaline phosphatase (FA), albumin, sodium, potassium, creatinine, urea, international normalized ratio (INR) and C-reactive protein (CRP).

Elastography measurements impulse response (VCTE) and controlled attenuation parameter (CAP)

VCTE was performed using a FibroScan ® device (EchoSens, Paris, France), in the right liver lobe under fasting conditions of the patient for more than 4-6 hours and in the supine position, with the right arm in maximum abduction, through an intercostal approach. In each patient, we tracked 10 valid LS measurements using the M probe (standard probe – 3.5 MHz transducer frequency) or the XL probe (2.5 MHz transducer frequency). The M and XL probes were used according to the EFSUMB recommendation on the selection of M and XL probes. The median value of 10 valid LS measurements was calculated and the results were expressed in kilopascals (kPa). Reliability measurements were defined as the median value of 10 valid LS measurements with a range interval interquartile /median ratio (IQR/M) <30%.

2D-SWE Elastography and Attenuation Imaging (ATI)

RH assessment by 2D-SWE was performed using the Multi-Frequency Slim Face Convex PVI-475BX (i8CX1) 4 probe. All measurements were performed under fasting conditions for at least 4 hours, with the patient in a supine position, right arm in maximum abduction, through an intercostal approach, in the right lobe of the liver.

Patients were asked to hold their breath for a few seconds without deep inspiration before holding their breath. Reliable LS measurements were defined as the median value of 5 measurements performed in a homogeneous area of the liver parenchyma, with an IQR/M <30%. For attenuation imaging (ATI) we followed the acquisition protocol proposed by the manufacturer: patient supine, same intercostal window, probe perpendicular to liver surface.

Evaluation by CEUS-PAT

Ultrasonographic evaluations were performed using the LOGIQ E9 system (GE Healthcare, Chalfont St. Gilles-UK) and the C1-6 probe. Each subject underwent a contrast-enhanced ultrasound (CEUS) evaluation using a contrast agent called SonoVue (Bracco SpA, Milan, Italy), using half the dose. Information obtained within the first 30 s of the liver scan was stored as raw data on a hard disk. The examination was performed with the patients in the left lateral position with the right hand raised above the head. Images showing liver parenchyma and right kidney on the same screen were used for analysis. The software provided the arrival time parametric imaging (AtPI) values of the contrast agent, the method called CEUS-PAT. The ratio of AtPI values for kidney and liver was then

calculated. A parametric arrival time ratio was calculated as the ratio of the contrast arrival time in the kidney to the contrast arrival time in the liver.

Statistical analysis

Statistical tests were performed using R software, V.2.5.1 (R Development Core Team, Vienna, Austria), MedCalc (v. 19.3.1, Ostend, Belgium) and IBM SPSS Statistics v. 20.0.0 (New York, NY, USA).

The Kolmogorov-Smirnov test was used to test the distribution of numerical variables. Qualitative variables were presented as numbers and percentages. Parametric tests (t-test, ANOVA) were used to assess differences between normally distributed numerical variables; and non-parametric tests (Mann- Whitney or Kruskal -Wallis tests) for variables with non-normal distribution. The Chi-square ($\chi 2$) test was used to compare proportions expressed as percentages. The Pearson correlation coefficient (r) was used to assess the association between two variables. A generalized linear model (GLM) was applied to analyze the significance of clinical and paraclinical factors associated with CEUS-PAT results; a subsequent univariate and multivariate analysis was also performed.

Receiver operating characteristic (ROC) analysis was used to evaluate the performance of the method. Youden 's J statistic was further used to determine the optimal *cut-off value* as a diagnostic test for liver cirrhosis. In all statistical analyses, the confidence level was 95% with a corresponding significance level of 5%.

3. RESULTS

3.1. Screening of liver fibrosis and steatosis in a large cohort of patients with type 2 diabetes using VCTE and CAP

A total of 776 patients were examined using VCTE and CAP during the study period. 242 of them were excluded, and the study cohort included 534 patients with T2DM. Regarding fibrosis severity, according to VCTE measurements, 72.6% (388 patients) had no mild fibrosis – F0 and F1, 7.8% (42) had F2, 11.4% (61) F3 and 8 .2% (43 patients) F4. Patients with at least advanced fibrosis were found more frequently in obese patients than in overweight and normal-weight patients, 23.3% vs. 14.1% vs. 12%, $p < 0.001$. Absence of steatosis was strongly correlated with normal weight ($r = 0.90$, $p = 0.01$); Mild steatosis was correlated with overweight ($r = 0.69$, $p < 0.0001$), and severe steatosis was strongly correlated with obesity ($r = 0.91$, $p < 0.0001$). CAP values increased significantly with weight status. For the entire cohort, we found that female gender ($p = 0.02$), BMI ($p = 0.03$), waist circumference ($p < 0.0001$), elevated AST levels ($p = 0.03$), total cholesterol ($p = 0.01$), triglycerides ($p < 0.0001$), blood glucose ($p = 0.0009$) and high MRH ($p = 0.0006$) were

associated with severe steatosis. MRHs increased with increasing BMI and waist circumference. For the entire cohort, we found that BMI ($p < 0.0001$), waist circumference ($p = 0.0002$), an elevated AST level ($p < 0.0001$), severe steatosis (0.0007) HbA1c ($p = 0.04$) and higher CAP values ($p = 0.002$) were the factors associated with advanced fibrosis (F3 and F4). In multivariate analysis, only AST was independently associated with advanced fibrosis.

3.2. Quantification of hepatic steatosis and fibrosis using a new system implemented in an ultrasound machine

A very strong positive correlation was found between the RH values obtained by VCTE and 2D-SWE: $r=0.88$, $p<0.0001$ and between the steatosis evaluation coefficients (CAP vs. ATI), $r=0.81$, $p< 0.0001$.

Regarding steatosis, the best cut-off values of ATI were: for S≥1 - 0.79 dB/cm/MHz (AUROC 0.88; Se=71.7%; Sp =95.4 %, VPP=97.1, VPN=61.8%), for S3 - 0.86 dB/cm/MHz (AUROC 0.94; Se=91.6%; Sp =87.5%, NPV=90,3, PPV=89.2) For fibrosis, the best cut -off values were: F≥2, 7.9 kPa, Se=69.2%, Sp =95.2%, VPN=83, 3%, VPP=90.0%, AUROC=0.89, $p<0.0001$.

3.3. Dynamics of liver stiffness values by elastography pulse in patients with HCV cirrhosis undergoing interferon-free treatment

Of 225 subjects, reliable measurements by VCTE were obtained in 93.7%, so the final analysis included 211 patients (116 women and 95 men), with a mean age of 59.2 ± 8.7 years, BMI 27.4 ±4.3 kg/m^2.

At EOT, the mean LS values of the study group decreased significantly compared to baseline: 23.5 ± 13.3 kPa (95% CI: 21.2 - 23.9) versus 26.4 ± 11.7 kPa (95% CI: 24.9 - 27.5), $p=0.01$.

Almost 60% of patients (59.2% - 125/211) showed a decrease of more than 10% in LS values; in 24.1% (51/211) LS values remained stable, while in 16.4% (35/211) of cases, LS values increased.

Evaluating other factors that may also be associated with a decrease in LS values, in both univariate and multivariate analysis, ALT was significantly correlated with the decrease in LS ($p=0.001$). Factors such as BMI, age or AST level were not correlated with decreased LS ($p=0.6$, $p=0.76$, $p=0.53$, respectively). Three-quarters of patients (75.2% - 128/170) had a greater than 10% decrease in LS values at 12 weeks post-EOT compared to baseline; in 13% (22/170) of cases, LS values remained stable, while in 11.8% (20/170) of cases they increased.

3.4. Contrast-enhanced ultrasound (CEUS) and parametric arrival time imaging (PAT) a non-invasive diagnostic tool for liver cirrhosis

The study included 64 subjects, 37 patients with liver cirrhosis and 27 healthy volunteers, with a mean age of 58.98 ± 8.90, who were predominantly male (56.3%). In the liver cirrhosis group, the majority were alcoholic cirrhosis (ALD) (35.1%), followed by MASLD (27.1%), HCV (21.6%), and HBV (16.2%).

Significant differences were found between the group of healthy volunteers and the group with liver cirrhosis by comparison, the group with liver cirrhosis had lower values of albumin ($p < 0.0001$) and haemoglobin ($p = 0.02$), higher values of CRP (0.0007), ALT ($p = 0.02$) and AST ($p = 0.03$).

Based on the Youden index, the optimal threshold value for detecting liver cirrhosis was a kidney-to-liver AtPI ratio of more than 0.7, with an AUROC of 0.98, $p < 0.001$, Se = 89.19%, Sp = 100 %, PPV = 100% and NPV = 87.1%.

The correlation between CEUS-PAT and VCTE was strong and highly significant, with a Spearman coefficient $r = 0.81$ ($p < 0.0001$).

bootstrap method was applied to determine the confidence interval of differences in CEUS-PAT between cases and controls. Also, the confidence interval for the ROC curve of CEUS-PAT was reestimated.

4. DISCUSSIONS

The problem of MASLD in the general population and also in certain categories, such as patients with type 2 diabetes mellitus (T2D) or metabolic syndrome, has become the subject of extensive research in recent decades. Several papers and meta-analyses have emphasized the importance of MASLD in T2D patients, but the medical community is not yet ready to start screening for MASLD and MASH in all diabetic patients. This paper wants to highlight, for all medical personnel involved in this field (diabetologists, specialists in internal medicine and hepatologists), that this pathological condition is quite common in daily practice. There appears to be an association between a high amount of body fat in T2DM and the incidence of MASLD. Therefore, we should insist on lifestyle changes through diet and physical activity in this category of patients. Published data show that the prevalence of MASLD varies between 42.6 and 69% in T2D patients, whereas a previous study from our area showed a prevalence of MASLD in T2D patients of up to 87.1%. The prognosis for these patients is different if they have only simple steatosis—nonalcoholic fatty liver—or if they have already developed MASH, early MASH (no or mild fibrosis), fibrotic MASH (significant/advanced fibrosis), or MASH cirrhosis.

The severity of liver fibrosis is the main prognostic factor in patients with MASLD. When we assessed the severity of fibrosis by VCTE in our patient group, based on the cutoffs proposed by Eddowes et al., we found that 19.4% of these patients were at high risk of developing severe fibrosis (11.3% had F3 and 8.1% F4), thus having advanced chronic compensated liver disease (cACLD) and being at risk of portal hypertension, decompensated liver disease or the development of hepatocellular carcinoma. As nearly 20% of diabetic patients are at risk of cACLD, it seems reasonable to screen all diabetic patients by liver elastography.

Non-invasive techniques are very attractive for the dynamic assessment of liver fibrosis over time because they are repetitive, traumatic, inexpensive and well-accepted by patients. But can we evaluate only fibrosis, or also other changes such as inflammation and possibly steatosis through VCTE? To partially answer this question, we designed this study, in which, after 12 weeks of treatment, we assessed LS in virally responsive HCV patients. Because the follow-up period was short, only 12 weeks after treatment, improvement in inflammation, not regression of fibrosis, was most likely responsible for the decrease in LS. Therefore, elastographic monitoring of cirrhotic patients who have achieved SVR should start from values measured at EOT or 12 weeks after EOT (when SVR is assessed), so that we can find out how much fibrosis has decreased in this disease advanced liver disease. Several studies have been published on LS values assessed by VCTE during and after treatment in both chronic HCV and HBV infection. In a study evaluating LS values by VCTE before and after interferon-based treatment in a cohort of 76 HCV patients, it was found that in the 55 patients with SVR, LS values at EOT were significantly decreased compared to initial value (6.8 ± 4.9 kPa vs. 9.5 ± 6.9 kPa, p=0.04). The decrease in LS values was maintained in the subgroup of patients who were followed up 3 years later. The short duration of IFT in compensated HCV cirrhosis (12 weeks) makes a decrease in fibrosis unlikely; most likely, the decrease in LS values compared to baseline was the result of attenuation of inflammation. We believe that EOT is an appropriate time to start fibrosis monitoring by VCTE, every 6 months. On the other hand, all cirrhotic patients should be monitored by ultrasound every 6 months for HCC, bearing in mind that this risk is still present despite viral eradication.

Over time, different study groups have investigated how CEUS can be applied in the diagnosis of liver fibrosis. This starts with the determination of the red ratio, done utilizing external software. It was later developed by analyzing CEUS components by time-intensity curve (TIC) and arrival time in the hepatic vein, artery and portal vein. Previous studies have shown that CEUS can diagnose liver cirrhosis by studying the arrival time of the contrast agent in the hepatic vein, hepatic artery, portal vein, and liver parenchyma while measuring the respective red colour ratios. If a patient has advanced fibrosis, their circulation will be

impaired, so the arrival time of the contrast agent is shortened. Another explanation could be that the contrast agent remains in the sinusoidal space. One study group conducted a study of 23 patients with MASLD comparing the use of biopsy to assess liver fibrosis. They calculated the differences in arrival time between the hepatic vein, hepatic artery, and hepatic parenchyma, and their results are similar to ours, finding that CEUS can be used to rule out severe cases of fibrosis. Recently, Yoshimine et al conducted a study on 48 patients with biliary cholangitis based on the performance of CEUS with AtPI in the diagnosis of liver fibrosis. They calculated the arrival time by red ratio with external software. The AUROC values of CEUS with AtPi in diagnosing F2 and F3 were 0.77 and 0.92, similar to our CEUS-PAT-derived AUC of 0.98, with good performance for diagnosing liver fibrosis. The findings of our study suggest that CEUS-PAT is a highly accurate method for diagnosing liver cirrhosis of various etiologies, using a cut-off value of over 0.7, with an AUROC value of 0.98, and very high sensitivity, specificity, PPV and NPV. On the other hand, different liver elastography methods have also been shown to perform well in predicting liver cirrhosis. However, in general, their performances are lower than CEUS-PAT. CEUS-PAT had higher values in the cirrhotic group compared to the healthy volunteer group, similar to VCTE values. However, in CEUS-PAT, there was a small level of overlap between groups, but without any clinical relevance. Although CEUS has been shown over the years to be a good diagnostic tool for the evaluation of liver fibrosis, the novelty of our study is that we demonstrated that CEUS-PAT is a much faster method with better performance in the diagnosis of liver cirrhosis. The fact that we can measure the arrival time with the same ultrasound machine is an advantage, saving time for doctors. Even though the method has a higher cost than the usual elastography methods, it still saves time and money for the subjects, because the diagnostic algorithm of focal liver lesions induces much lower costs than computed tomography and magnetic resonance imaging.

5. CONCLUSIONS

- **a.** 60% of patients with diabetes and obesity were found to have severe steatosis, of which 19% were also found to have advanced fibrosis, results that support the need to implement an individualized screening program for the patient, depending on BMI.
- **b.** Univariate analysis in our study, female sex, BMI, waist circumference, elevated AST, total cholesterol, triglycerides, and elevated blood glucose values were associated with severe steatosis.
- **c.** Increased BMI, waist circumference, and severe steatosis were associated with advanced fibrosis.

d. Elastography on the *Aplio i800 system* is a reliable method for the non-invasive assessment of liver fibrosis with excellent feasibility (99.1%) and a very strong correlation with VCTE (r=0.88).

e. The threshold values for 2D-SWE for predicting F≥2 and F=4 are 7.9 kPa and 11.7 kPa, respectively.

f. ATI proved to be a very good method for quantifying steatosis, the correlation with CAP being strong (r= 0.81).

g. For the diagnosis of different degrees of steatosis with the help of ATI, we established the following threshold values : (a) for S 1, 0.79 dB/cm/ mHz ; (b) for S3, 0.86 dB/cm/ mHz.

h. Post-antiviral treatment, approximately 60% of subjects had a 10% decrease in LS, possibly explained by the decrease in the inflammatory process.

i. CEUS-PAT can be considered a new non-invasive method for the diagnosis of liver cirrhosis, the correlation with VCTE being r = 0.81.

j. The performance of CEUS-PAT for the diagnosis of liver cirrhosis proved very good, AUROC = 0.98 and PPV = 100%, at a *cut-off value* of 0.7, which makes it suitable for implementation.

k. Through the *Bootstrapping method*, we resampled the CEUS-PAT measurements, the results being similar to those from the conventional analysis, which reinforces the conclusions regarding the accuracy of the CEUS-PAT method.