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PhD THESIS

**THE MULTIPARAMETRIC ASSESSMENT OF DIFFUSE
CHRONIC LIVER DISEASE USING ULTRASOUND-BASED
TECHNIQUES**

A B S T R A C T

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ABSTRACT

GENERAL PART

Chronic liver diseases are a major cause of morbidity and mortality and represent a significant global health problem. Non-alcoholic fatty liver disease (NAFLD), hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol-related liver disease (ALD) are the most prevalent etiologies of CLD. Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in developed countries, with a prevalence of up to 35%, and is becoming a serious clinical concern.

Liver injury produces a sequence of inflammatory events that lead to chronic inflammation. Chronic inflammation is an active process that induces the development of liver fibrosis, eventually inducing the evolution to cirrhosis and hepatocellular carcinoma. As it marks a critical moment in the progression of CLD, liver fibrosis is regarded as one of the most significant prognostic factors. Hepatic steatosis, or fatty liver, is also a significant prognostic factor associated with CLD progression. Furthermore, the need for liver fat quantification and monitoring has increased due to the rapidly spreading obesity epidemic and the introduction of new therapeutics. Up until recently, liver biopsy was the only method for determining the presence of liver fibrosis, steatosis, and inflammation. However, liver biopsy is an invasive procedure with renowned disadvantages such as potential risks, high costs, and inter- and intra-operator variability of histologic assessments among pathologists.

However, significant improvements have been made over the past 20 years. Several non-invasive ultrasound-based techniques that assess the physical properties of the liver tissue were developed for evaluating the severity of CLD. Currently, the physical properties of the tissue can be evaluated using ultrasound-based elastography by analyzing the tissue interactions with acoustic energy. Non-invasive ultrasound-based liver elastography techniques for evaluating liver stiffness (LS) have become widely available. One of the first and the most validated elastographic method was Vibration-Controlled Transient Elastography (VCTE), followed by other techniques, such as Point Shear Wave Elastography (pSWE) and Two-Dimensional Shear Wave Elastography (2D-SWE).

In conventional gray-scale ultrasound, "bright liver" with "posterior beam attenuation" are the typical ultrasound signs of liver steatosis. The aforementioned indicators have led to the development of several quantitative ultrasound-based techniques for liver steatosis assessment: the backscatter coefficient, attenuation coefficient, the acoustic structure quantification, and speed of sound quantification.

In addition to liver fibrosis, numerous other factors influence LS. Among them, probably the most important and challenging to discriminate is necro-inflammation. In light of the fact that these clinical conditions frequently coexist, it is essential to evaluate whether the increased LS is attributable to fibrosis or inflammation. Shear wave dispersion is an ultrasound-based method that can be used as an indirect marker of liver viscosity. According to preliminary studies, this method might be useful for determining inflammation. Thus, with the development of these new techniques, a multiparametric ultrasound examination approach can be used when evaluating patients with CLD.

SPECIFIC PART

The current research aimed to evaluate the performance of the newly developed multiparametric ultrasound-based tools for measuring LS (2D-SWE PLUS), attenuation (Att PLUS), speed of sound (SSp PLUS), and viscosity (Vi PLUS) as surrogate markers of liver fibrosis, liver steatosis, and necro-inflammation. The aforementioned techniques were embedded in the same new ultrasound device – the Supersonic MACH® 30 US system (Hologic® SuperSonic® Imagine, Aix-en-Provence, France) and allow a simultaneous

quantification of these parameters. LS was also evaluated using a novel, innovative two-dimensional time-harmonic elastography technique (THE).

1. GENERAL OBJECTIVES

- (1) To establish the feasibility and performance of a new two-dimensional shear wave elastography technique with a new quality criterion (2D-SWE PLUS) to predict the presence and severity of liver fibrosis in patients with mixed CLD and in patients with NAFLD using VCTE as the reference.
- (2) To evaluate the feasibility, reproducibility, and performance of an innovative ultrasound-based technique (THE) in staging liver fibrosis using VTCE as the reference.
- (3) To establish the feasibility and performance of a new ultrasound-based technique, SSF PLUS, to diagnose and stage liver steatosis in patients with mixed CLD and in patients with NAFLD, using CAP as the reference.
- (4) To determine the feasibility and performance of Att PLUS to detect the presence of liver steatosis in patients with mixed CLD.
- (5) To evaluate the feasibility a novel ultrasound-based method for evaluating liver viscosity, to determine the normal range of liver viscosity values in healthy subjects and analyze the factors that influence them.
- (6) To determine the factors that influence liver viscosity measurements in a large cohort of CLD patients.

Considering that the primary objectives of the component studies of this thesis were the quantification of fibrosis, steatosis, and inflammation using quantitative multiparametric ultrasound-based techniques, the specific part was divided into three distinct chapters: ultrasound-based quantification of LS as a marker of liver fibrosis; ultrasound-based quantification of liver steatosis; and ultrasound-based quantification of viscosity as a marker of liver inflammation.

2. MATERIALS AND METHODS

Subjects

Before enrolling in the component studies of the present research, all subjects gave written consent to undergo elastographic measurements as well as clinical, ultrasonographic, and biological examinations. Firstly, all the patients included in this research underwent a complete abdominal ultrasound scan before liver elastography was performed, paying particular attention to the signs that might affect the LS measurements: the presence or absence of focal liver lesions, of biliary obstruction or portal vein thrombosis, of ascites, and of heart failure.

Vibration-Controlled Transient Elastography (VCTE) and Controlled Attenuation Parameter (CAP)

VCTE with CAP measurements were acquired using FibroScan® Compact 530 (EchoSens®, Paris, France) and were used as the reference method in our research. In order to differentiate between different degrees of steatosis and fibrosis, specific cut-off values were chosen according to the predominant pathology of the subjects included in the study.

Two-Dimensional ShearWave PLUS Elastography (2D-SWE PLUS)

2D-SWE PLUS measurements were acquired with a C6-1X curvilinear transducer using the UltraFast™ software available on the SuperSonic Mach 30 ultrasound system. The stability index tool (SI) is a new quality criterion developed by SSI. It originates from the temporal and spatial stiffness stability within the circular Q-Box. The IQR to the median ratio (IQR/M) of less than 30% and the SI of above 90% were used as measurement reliability criterions.

Viscosity PLane wave UltraSound (Vi PLUS)

Vi PLUS is a new technique implemented in the SuperSonic Mach 30 that displays data about tissue shear wave dispersion. Vi PLUS mode is available simultaneously with 2D-SWE PLUS mode. The Q-Box is duplicated on both SWE and Vi PLUS images allowing the stiffness and viscosity quantification in the same place and at the same time. The changes in the shear waves speed between frequencies are expressed quantitatively in pascal-second (Pa·s) and displayed qualitatively in the form of a color-coded map. As Vi PLUS and 2D-SWE PLUS are combined in the same measurement, the same protocol and the same quality criteria were used.

The Attenuation Plane-Wave Ultrasound (Att PLUS) and The Sound Speed Plane-Wave Ultrasound (SSp PLUS)

SSp PLUS is a novel method that allows the assessment of the intrahepatic speed of sound, reflecting fat content, an important marker for the detection of liver steatosis. Sound speed measurements are expressed in m/s. By measuring the decrease in amplitude of the ultrasound waves as they propagate through the liver tissue as a function of frequency, the Att PLUS measures the attenuation of the ultrasound beam across a ROI as a marker of steatosis. The Att PLUS values are expressed in dB/cm/MHz. Att PLUS and SSp PLUS methods are available in B-mode, on a live image, as a single acquisition. Reliable results were defined as the median value of five valid measurements, with an IQR/M below 30%.

Two-Dimensional Time-Harmonic Elastography (THE)

The THE system (GAMPT mbH, Merseburg, Germany) uses a multiharmonic waveform produced by the vibration bed to generate the shear wave excitation. A clinical ultrasound scanner (SonixMDP, Ultrasonix, Scottsdale, AZ, USA) was used for shear wave acquisition. After the multifrequency time harmonic waveform was produced, and measurements were acquired, full color-coded elastography maps were displayed. An ROI was then selected in the most homogeneous area. A reliable result was considered the median value of 10 measurements with an IQR/M below 30%.

Statistical analysis

Microsoft Office Excel 2019 (Microsoft for Windows) and MedCalc Version 19.4 (MedCalc Software Corp., Brunswick, ME, USA) were used to perform the statistical analysis for the studies that assessed the techniques embedded in the SuperSonic Mach 30 Ultrasound device (2D-SWE PLUS, Att PLUS, SSp PLUS, and Vi PLUS). The THE system was analyzed using R software version 2.5.1 (R Development Core Team, Vienna, Austria) and IBM SPSS Statistics version 17 (IBM Statistics, Chicago, IL, USA).

3. RESULTS

3.1. QUANTIFICATION OF LIVER STIFFNESS AS A MARKER OF LIVER FIBROSIS

3.1.1. Ultrasound-Based Quantification of Fibrosis with a New Software Considering Vibration-Controlled Transient Elastography as Reference in Patients with Chronic Liver Diseases

The first study to be published, entitled “Ultrasound-Based Quantification of Fibrosis and Steatosis with a New Software Considering Vibration-Controlled Transient Elastography as Reference in Patients with Chronic Liver Diseases”, was a monocentric cross-sectional study conducted in a tertiary department of Gastroenterology and Hepatology between March and August of 2020. 133 consecutive adult patients with chronic hepatopathies who volunteered to undergo LS and steatosis measurements using 2D-SWE PLUS, SSp PLUS, and using VCTE with CAP were enrolled. To discriminate between the stages of fibrosis using VCTE, the following cut-offs were considered: $F2 \geq 7$ kPa; $F3 \geq 9.5$ kPa and $F4 = 12$ kPa.

Valid LS measurements were obtained in 97.7% (130/133) of patients by VCTE, and by 2D-SWE PLUS in 93.2% (124/133) of patients. In terms of technical success rates, there was no statistically significant difference between VCTE and 2D-SWE PLUS ($p = 0.076$).

The Bland-Altman analysis revealed that, with the exception of individuals with little or mild fibrosis (F0-1), there are no significant differences between the mean LS values obtained by 2D-SWE PLUS and VCTE.

The results of the linear regression analysis revealed an excellent correlation between the values obtained by 2D-SWE PLUS and VCTE ($r = 0.92$, $R^2 = 0.85$, $p < 0.001$).

2D-SWE showed excellent performance in staging liver fibrosis in CLD patients as compared to the control method. The optimal 2D-SWE PLUS cut-off values using VCTE as the reference method, based on the Youden index, were 6.8 kPa for $F \geq 2$ (AUROC = 0.94), 8.4 kPa for $F \geq 3$ (AUROC = 0.95) and 11 kPa for $F4$ (AUROC = 0.95).

3.1.2. Quantification Of Liver Stiffness As A Marker Of Liver Fibrosis In Patients With Non-Alcoholic Liver Disease: A “Real-Life” Cohort Study

A monocentric cross-sectional study was conducted in a tertiary department of gastroenterology and hepatology over the course of four months (October 2020 to February 2021). 215 NAFLD patients were enrolled. In the same session, ultrasound-based multiparametric measurements were performed in all patients utilizing 2D-SWE PLUS, Att PLUS, Sound Speed Plane-wave Ultrasound, Vi PLUS, and using VCTE with CAP.

In this study, the following VCTE cut-off values were utilized for liver fibrosis assessment: $F2 \geq 8.2$ kPa, $F3 \geq 9.7$ kPa, and $F4 = 13.6$ kPa, as recommended by Eddowes et al. (2019).

Valid LSM were achieved in 95.8% (206/215) of cases using 2D-SWE PLUS. Patients with unreliable measurements had significantly higher mean abdominal circumference and BMI (kg/m^2) values than those with reliable results (123.18 ± 4.86 vs. 108.74 ± 11.30 , $p < 0.0001$ and 35.77 ± 6.43 kg/m^2 vs. 31.27 ± 5.56 kg/m^2 , $p = 0.01$). There were no significant differences in the feasibility of VCTE and 2D-SWE PLUS ($p = 0.14$).

The correlation between LSM acquired by 2D-SWE PLUS and VCTE was found to be excellent ($r = 0.89$, $R^2 = 0.79$, $p < 0.0001$).

2D-SWE PLUS showed excellent performance (AUROC = 0.91) for identifying significant fibrosis in NAFLD patients, with an optimal cut-off value of 7 kPa (Se = 90.9%, Sp = 91.8%, PPV = 68.2%, NPV = 98.1%).

3.1.3. Performance of a Noninvasive Time-Harmonic Elastography (THE) Technique for Liver Fibrosis Evaluation Using Vibration-Controlled Transient Elastography (VCTE) as Reference Method

To date, all ultrasound-based elastography methods generate small elastograms that reflect only a small portion of the entire organ, which may restrict the diagnostic accuracy of elastography in the evaluation of diffuse chronic liver disease. Consequently, the objective of this study was to evaluate an innovative two-dimensional time-harmonic elastography technique that provides complete field elastograms. A total of 165 subjects (healthy subjects and patients with various CLD) were included in the final analysis. The mean LS values in healthy subjects were significantly lower by VCTE than by THE: 5 ± 1.27 kPa, $p < 0.0001$ vs. 6.94 ± 0.75 kPa. To avoid bias in evaluating THE performance for predicting liver fibrosis, we eliminated healthy individuals from further analysis. THE system had significantly higher values compared to VCTE in F0 - F1 stages ($p < 0.0001$), but significantly lower LS values compared to VCTE in F4 ($p < 0.0001$).

A robust, significant correlation ($r = 0.82$) was found between LS values obtained by using VCTE and THE, $p < 0.0001$.

The highest accuracy was obtained for liver cirrhosis, with an AUROC of 0.90 (95 % CI 0.82 - 0.93) and it decreased slightly for significant fibrosis. For significant fibrosis ($\geq F2$), the optimum cut-off value was 7.58 kPa, NPV = 76.7 %, PPV = 82.9 %, Se = 81.8 %, Sp = 77.9 %.

In the univariate analysis, higher GGT ($p = 0.002$), ALP ($p = 0.04$), AST ($p = 0.0004$), age above 60 ($p < 0.0001$) and female gender ($p < 0.0001$) were factors that influenced THE values.

Excellent interobserver reproducibility was demonstrated by the ICCs between the three types of examiners (novice, elastography expert, and ultrasound expert). Overall inter-examiner agreement was excellent: 0.94

3.2. ULTRASOUND-BASED QUANTIFICATION OF LIVER STEATOSIS

3.2.1. Ultrasound-Based Quantification Of Liver Steatosis Using A New Software (SSp PLUS) Considering Controlled Attenuation Parameter As Reference In Patients With Chronic Liver Diseases

An important part of the study “Ultrasound-Based Quantification of Fibrosis and Steatosis with a New Software Considering Vibration-Controlled Transient Elastography as a Reference in Patients with Chronic Liver Diseases” aimed to analyze the performance of a new ultrasound-based technique in detecting the presence of hepatic steatosis using CAP as a control method. To discriminate between the stages of steatosis, the cut-off values for CAP used in our study have been those recommended by Eddowes et al. (2019): 274 dB/m for the presence of liver steatosis. Using the CAP cut-off value of 274 dB/m, 75.4% (92 patients) had steatosis.

Linear regression analysis demonstrated a strong correlation between values obtained by using SSp PLUS and CAP ($r = -0.70$, $R^2 = 0.50$, $p < 0.001$).

The optimum SSp PLUS cut-off value based on the Youden index for predicting the presence of liver steatosis using a CAP cut-off value of >274 dB/m was ≤ 1537 (AUROC = 0.82, Se = 75%, Sp = 83.3 %, NPV = 53.1%, PPV = 93.2%).

3.2.2. Quantification Of Liver Steatosis (SSp PLUS And Att PLUS) In Patients With Non-Alcoholic Liver Disease: A “Real-Life” Cohort Study

Being the most common liver disease in developed countries, with a prevalence of up to 35%, NAFLD is steadily becoming a serious clinical concern. As the evaluation of liver steatosis is a crucial diagnostic point in NAFLD patients, an important part of the study “Quantification of Liver Fibrosis, Steatosis, and Viscosity Using Multiparametric Ultrasound in Patients with Non-Alcoholic Liver Disease: A “Real-Life” Cohort Study” is intended to analyze the feasibility and performance of the two new ultrasound-based methods (SSp PLUS and Att PLUS) for predicting the presence of liver steatosis in a cohort of patients with NAFLD, using CAP as the control method. In this study, for discriminating between different stages of liver steatosis, the cut-off values recommended by Petroff et al. (2021) were utilized: S1: 294 dB/m, S2: 310 dB/m, and S3: 331 dB/m, respectively. The cut-off value of 310 dB/m by CAP was considered as suggestive for significant steatosis (S2–S3).

Valid liver steatosis measurements were obtained in 98.1% (211/215) by using Att PLUS and SSp PLUS. No significant differences between the feasibility of CAP and Att/SSp PLUS ($p = 0.98$) were found.

The values obtained by using SSp PLUS correlated better with CAP values than Att PLUS: ($r = -0.74$, $p < 0.001$) vs. ($r = 0.45$, $p < 0.001$). The optimum Att PLUS cut-off value for predicting S2-S3 steatosis was 0.5 dB/cm/MHz (AUROC = 0.72) . The optimum SSp PLUS cut-off value for predicting S2-S3 steatosis was <1524 m/s (AUROC = 0.88).

Univariate and multivariate statistical analyses were employed to assess the associations between SSp PLUS values and the following parameters: ALT, AST, triglyceride levels, cholesterol levels, BMI, abdominal circumference, Att PLUS values, and CAP values.

Univariate analysis showed that abdominal circumference ($p < 0.001$), BMI ($p = 0.002$), Att PLUS values ($p < 0.001$) and CAP values ($p < 0.001$) were independently associated with SSp PLUS values.

The multivariate regression analysis revealed that only CAP values ($p < 0.001$) and abdominal circumference ($p < 0.001$) were independently associated with SSp PLUS values.

Subjects with diabetes mellitus ($n = 56$) had significantly lower mean values than those without (1510.3 ± 25.1 m/s vs. 1529.7 ± 28.4 m/s, $p < 0.0001$). However, no significant differences in SSp PLUS mean values were identified between participants with arterial hypertension ($n = 128$) and those without (1522.9 ± 28.5 m/s vs. 1526.7 ± 29.3 m/s, $p = 0.358$).

3.3. ULTRASOUND BASED QUANTIFICATION OF LIVER VISCOSITY

3.3.1. The Non-Invasive Ultrasound-Based Assessment of Liver Viscosity in a Healthy Cohort

This study sought to assess the normal range of liver viscosity values in healthy subjects and analyze the factors that influence them. 131 consecutive subjects with healthy livers were included in this prospective monocentric study, which was carried out in a tertiary gastroenterology and hepatology center between October 2019 and October 2021.

Valid measurements were obtained in 93.9% (123/131) of subjects using 2D-SWE & Vi PLUS. The mean abdominal circumference values of patients with unreliable measurements were significantly higher than of those with reliable measurements (95.7 ± 56.26 cm vs. 84.70 ± 12.06 cm, $p = 0.0115$), whereas there were no significant differences in BMI mean values (25.35 ± 2.73 kg/m² vs. 24.02 ± 3.40 kg/m², $p = 0.2410$).

Subjects with healthy livers ($n = 123$) had mean liver Vi PLUS values of 1.57 ± 0.20 Pa·s for females and 1.62 ± 0.21 Pa·s for males, respectively. There were no significant differences between the mean values of Vi PLUS ($p = 0.1872$). The mean values of Vi PLUS increased with each decade of age. All other subgroups had significantly higher mean values than the 18-30 age subgroup (all $p > 0.05$).

According to BMI (kg/m²), 73/123 (59.4%) subjects were of normal weight ($\text{BMI (kg/m}^2\text{)} < 25$), whereas 50/123 (40.6%) were overweight ($25 \leq \text{BMI (kg/m}^2\text{)} < 30$). Normal-weight subjects had significantly lower mean Vi PLUS values (1.53 ± 0.19 Pa·s) than overweight subjects (1.67 ± 0.19 Pa·s) ($p = 0.0001$).

In univariate regression analysis, the following parameters were associated with Vi PLUS values: abdominal circumference ($p < 0.001$), BMI ($p < 0.001$), age ($p < 0.001$), LS values by 2D-SWE ($p < 0.001$) and LS values by VCTE ($p < 0.001$), respectively. Multivariate regression analysis showed that the model including BMI ($p = 0.0023$), age ($p = 0.0043$), and LS values by 2D-SWE ($p < 0.0001$) was associated with Vi PLUS values. Also, a moderate correlation between Vi PLUS values and LS values by 2D-SWE ($r = 0.66$, $p < 0.0001$) was obtained in normal subjects.

3.3.2. Quantification of Liver Viscosity (Vi PLUS) in Patients with Non-Alcoholic Liver Disease: A “Real-Life” Cohort Study

Due to the significant differences in prognosis, it is essential to differentiate between simple steatosis and steatohepatitis (NASH) in individuals with NAFLD. Therefore, a part of the study “Quantification of Liver Fibrosis, Steatosis, and Viscosity Using Multiparametric Ultrasound in Patients with Non-Alcoholic Liver Disease: A “Real-Life” Cohort Study” sought to evaluate the liver viscosity in NAFLD patients by using Vi PLUS and to analyze the factors that influence these measurements.

Univariate and multivariate statistical analyses were employed to investigate the relationships between the Vi PLUS values and the following parameters: abdominal circumference, BMI, ALT, AST, LS values obtained by VCTE, and LS values acquired by 2D-SWE PLUS. The univariate regression analysis displayed an independent association between Vi PLUS measurements and LSM obtained using both techniques, 2D-SWE PLUS and VCTE ($p < 0.001$), BMI ($p < 0.001$), abdominal circumference ($p < 0.001$). Neither AST ($p = 0.62$) nor ALT ($p = 0.49$) were statistically linked with Vi PLUS values obtained in NAFLD

patients. In multivariate regression analysis, BMI ($p < 0.0001$) and LS by 2D-SWE PLUS ($p < 0.0001$) were independently associated with Vi PLUS values.

3.3.3. Factors That Influence Ultrasound-based Viscosity (Vi PLUS) Measurements In Chronic Liver Disease – preliminary results

This study aimed to evaluate liver viscosity and to analyze factors influencing Vi PLUS measurements in a large cohort of patients with CLD. 668 patients referred to our department for liver fibrosis assessment were included. Reliable viscosity measurements were obtained in 93.5% (625/668) of the included subjects using Vi PLUS.

Vi PLUS mean values were higher in subjects with CLD than values obtained in the previous study that involved normal subjects (1.59 Pa·s), independent of the etiology. Mean Vi PLUS values were significantly higher in subjects with ALD (2.80 Pa·s) compared to HCV (2.14 Pa·s, $p < 0.0001$), HBV (1.84 Pa·s, $p < 0.0001$), NAFLD (1.97 Pa·s, $p < 0.0001$), mixed etiology (2.37 Pa·s, $p = 0.0327$) subjects, or subjects with others or unknown etiologies ($p = 0.0001$). No significant differences were found between mean Vi PLUS values in ALD subjects compared to those with autoimmune hepatitis (AH) ($p = 0.1209$). No significant differences were found between mean Vi PLUS values in AH subjects and any other etiology ($p > 0.05$). Vi PLUS mean values were significantly higher in subjects with HCV compared to HBV ($p = 0.0001$) and NAFLD subjects ($p = 0.011$). No significant differences were found between HBV and NAFLD subjects ($p = 0.0615$). Significantly higher values were also found in subjects with mixed etiology compared to those with HBV ($p < 0.0001$) and NAFLD ($p = 0.0001$).

In univariate regression analysis, Vi PLUS measurements were independently associated with: LS values obtained by 2D-SWE ($p < 0.001$) and VCTE ($p < 0.001$), BMI ($p < 0.001$), abdominal circumference ($p < 0.001$), age ($p < 0.001$), AST ($p < 0.001$), ALT ($p = 0.009$), the presence of diabetes mellitus ($p < 0.0001$) and the presence of arterial hypertension ($p < 0.001$), respectively. In multiple regression analysis, the model including: abdominal circumference ($p < 0.0001$), AST values ($p = 0.0338$), LS by 2D-SWE ($p < 0.0001$) and VCTE ($p = 0.0009$), was associated with Vi PLUS measurements.

4. DISCUSSIONS

Liver fibrosis was found to be the most important prognostic factor in CLD patients. Liver biopsy is still considered the gold-standard method for assessing liver fibrosis. However, due to its invasiveness and its well-recognized drawbacks, the development of quantitative non-invasive techniques for the assessment of liver fibrosis is of great importance.

The first question to be answered in this research concerned the feasibility and performance of a new elastography software embedded in the SuperSonic Mach 30 system in assessing fibrosis in patients with various CLD. In order to accomplish this, we conducted two studies: the first aimed to assess fibrosis and steatosis using two new US-based techniques in a cohort of patients with mixed CLD, and the second aimed to evaluate a cohort of patients with NAFLD using a multiparametric approach.

Numerous studies have demonstrated the efficacy of the old 2D-SWE technique from SSI in assessing liver fibrosis, but none have applied the newly introduced quality criterion, the stability index (SI). However, by raising the quality standard, the introduction of a new parameter, although it confers more reliable results, may decrease the feasibility of a method. In the first two studies published during our research, the results showed that using the 2D-SWE technique, with the new quality criterion, has an excellent feasibility of 95.8% (206/215) in NAFLD patients and of 93.2% (124/133) in patients with mixed CLD. Studies indicate that the previous 2D-SWE technique, which lacked the new accuracy parameter, had a similar degree of feasibility, ranging from 90 to 98 percent.

The study that analyzed a cohort of mixed CLD patients revealed an excellent correlation between the LS measurements obtained by 2D-SWE PLUS and VCTE ($r = 0.92$). The optimal 2D-SWE PLUS cut-off values were: 6.8 kPa for $F \geq 2$, 8.4 kPa for $F \geq 3$ and 11 kPa for $F4$. The estimated cut-off values are highly accurate for ruling in $F2$ (PPV = 88.7%) and ruling out $F4$ (NPV = 98%), similar to the findings of other published studies. In the study

that analyzed a cohort of NAFLD patients, the linear regression analysis showed an excellent correlation between the LS values obtained by 2D-SWE PLUS and VCTE ($r = 0.89$). In this study, the calculated 2D-SWE PLUS cut-off value for significant fibrosis ($F \geq 2$), using VCTE as the reference was 7 kPa.

To date, all ultrasound based elastography techniques generate small-sized elastograms that represent only a fraction of the whole organ, which may limit the diagnostic accuracy of elastography in the assessment of diffuse chronic liver disease. Therefore, a part of our research aimed to evaluate a novel innovative two-dimensional time-harmonic elastography technique. Using continuous multifrequency range shear waves generated by an external vibration device integrated into the patient bed, THE obtains full field elastograms of the liver tissue in the same manner as MRI elastography. The results showed a direct, significant, and strong correlation ($r = 0.82$) between LS assessed by VCTE and THE, $p < 0.0001$, AUROC was highest for F4 - 0.90, highlighting the value of this method in the diagnosis of liver cirrhosis. The method also showed good intra-, inter-observer reproducibility, and excellent feasibility. However, in addition to its strengths, this method requires additional equipment that is quite complex, difficult to use, and time-consuming. Therefore, the role of this method in usual clinical practice is limited.

Liver steatosis, or fatty liver, is also a significant prognostic factor associated with CLD progression. Because of the rapidly spreading obesity pandemic and the emergence of various NAFLD therapies, early diagnosis of liver steatosis has become critical for providing a timely and effective follow-up strategy for preventing liver disease development. Liver biopsy is the gold-standard for evaluating liver steatosis. However, it is understandable that in the case of an endemic disease not every patient with NAFLD or suspected of NAFLD can undergo liver biopsy, and certainly not repeatedly during follow-up. Therefore, several non-invasive ultrasound-based imaging techniques have been developed, CAP became the most widely used technique for first-line steatosis assessment and has been recommended by guidelines as an accurate technique for detection and grading liver steatosis. In recent years, several ultrasound device manufacturers have incorporated software capable of quantifying liver steatosis in addition to fibrosis.

In our research, two new techniques that use different physical principles were analyzed: Att PLUS and SSp PLUS. Att PLUS, which uses the attenuation coefficient to evaluate liver steatosis, showed an excellent feasibility of 98.1% and correlated moderately with the control method used in our study: $r = 0.46$. Several studies that analyzed techniques that use the attenuation coefficient for steatosis quantification were published. Measurements obtained using ATI by Canon and UGAP by GE strongly correlated with the control method used ($r = 0.75$ and $r = 0.81$). Jeon et al. (2021) showed that TAI by Samsung was also significantly correlated with the controlled method ($r = 0.65$). ATT by Hitachi, however, moderately correlated with the control method: $r = 0.50$. Our results show that Att PLUS has correlated moderately with the reference method used ($r = 0.45$).

The SSp PLUS was the second method for evaluating LS that was analyzed in our research. SSp PLUS quantifies liver steatosis by estimating the intrahepatic speed of sound, exploiting a different physical property of ultrasound than previously described approaches. A study published by Diguardi et al. (2019) evaluated the ability of a similar concept method that estimates the intrahepatic speed of sound to assess liver steatosis. A strong correlation ($R^2 = 0.73$) was observed between the speed of sound readings and MRI-PDFF when MRI-PDFF was used as the control method. A cutoff value of 1.537 mm/s had a sensitivity of 80% and a specificity of 85.7% for detecting steatosis (S1-S3). We analyzed the feasibility and performance of SSp PLUS throughout 2 studies. In the first study in a mixed CLD cohort, we found a strong correlation between SSp PLUS values and the control method used ($r = -0.70$). The optimal SSp PLUS cut-off for predicting the presence of liver steatosis ($S > 1$) was 1537 m/s (AUROC 0.82), with a sensitivity and specificity of 75% and 83.3%, respectively. The second study evaluated SSp PLUS's performance and feasibility in NAFLD patients. SSp PLUS proved an excellent feasibility of 98.1%. SSp PLUS correlated strongly with the control method ($r = -0.74$). The optimal SSp cut-off value for predicting significant hepatic steatosis ($S > 2$) was 1524 m/s (AUROC 0.88), with 75.5 percent sensitivity and 93.4 percent specificity.

Concerning the quantification of steatosis, our research showed that among the techniques analyzed, SSp PLUS performed better in the identification and staging of hepatic steatosis, demonstrating a stronger correlation with the control method.

As previously discussed, LS is a widely recognized marker of liver fibrosis. Nevertheless, the level of inflammation can have a significant effect on shear wave velocity, resulting in increased LS measurements. In addition, determining the level of inflammation is crucial in a number of distinct pathological conditions. Due to the substantial differences in prognosis, it is essential to differentiate between simple steatosis and steatohepatitis (NASH) in patients with NAFLD. Several studies demonstrated that LS decreased significantly in ALD patients after cessation of alcohol consumption, while fibrosis stages assessed by liver biopsy remained largely unchanged. LS also decreases in sustained responders following IFN-based therapy in patients with chronic HCV. The same pattern was observed in patients with HBV who were initiated on antiviral therapy. These results imply that inflammation is a major contributor to stiffness in addition to fibrosis. When evaluating the elastography results, it is essential to differentiate between the inflammatory component and fibrosis. To overcome this limitation, ultrasound manufacturers have developed new assessment parameters for CLD patients.

Such a parameter is Vi PLUS, an imaging technique based on the dispersion of shear waves, which is regarded as a surrogate for necroinflammation. An important part of our research sought to evaluate this new parameter: the feasibility of the method was analyzed; the values in healthy individuals were determined; and the factors influencing the obtained values were analyzed. Using the same quality criteria as in 2D-SWE, Vi PLUS had an excellent feasibility of 93.9%. Subjects with healthy livers had a mean viscosity value of 1.59 Pa·s. Therefore, a Vi PLUS measurement of approximately 1.59 Pa·s is indicative of a healthy liver, without fibrosis and inflammation. The mean values of Vi PLUS increased with age but were unaffected by gender. Regardless of the underlying cause, the viscosity values obtained from CLD patients were significantly higher than those from the healthy cohort. Patients with ALD had the highest values (2.8 Pa·s), which may be due to the significant inflammation and congestion observed in patients with active alcohol consumption. The univariate regression analysis shows that LS has a strong and significant effect on viscosity values. Also, high BMI seem to be associated with high viscosity values. A particular situation was observed in the case of transaminases. In the cohort of patients with mixed chronic liver diseases, the elevated level of transaminases influenced Vi Plus results, however, this was not observed in the cohort of patients with NAFLD. To gain a better understanding of the various factors that affect liver viscosity, additional research on patients with chronic hepatopathies, using liver biopsy as a reference standard, is required.

Implementing the aforementioned techniques within the same ultrasonographic device enables the simultaneous evaluation of fibrosis, steatosis, and inflammation, allowing a **multiparametric ultrasound-based** evaluation of CLD patients. NAFLD patients could benefit most from this ultrasound-based multiparametric approach. The evaluation of steatosis is the essential first step in establishing the diagnosis of NAFLD. Standard ultrasound is an excellent method to detect patients with moderate steatosis. However, quantitative techniques may improve the detection of mild steatosis. The differentiation of simple steatosis from steatohepatitis with necroinflammation (NASH) is an additional important step. Finally, being the most significant prognostic factor, fibrosis must be evaluated and monitored in this group of patients. In our research, all methods proved excellent feasibility, 2D-SWE and SSp PLUS proved useful to evaluate and stage liver fibrosis and steatosis in the NAFLD patient cohort. Regarding Vi PLUS, additional studies are needed to establish the usefulness of this method in differentiating NASH from simple steatosis.

Early diagnosis of CLD permits the initiation of specific measures to improve survival. Thus, recently, several approaches related to population screening have been considered. Regarding CLD screening, the recommendations of the guidelines are still under debate. The European Associations for the Study of the Liver, Diabetes, and Obesity recommends screening of hepatic steatosis in populations at risk using conventional ultrasound. Moreover, populations at risk must also be screened for fibrosis. VCTE or serum biomarkers are widely available, well-validated, and widely accepted for

fibrosis screening. However, ultrasound-based noninvasive techniques have a higher sensitivity and specificity for detecting steatosis than conventional ultrasound, and a similar sensitivity and specificity for detecting fibrosis as VCTE. With the benefit of simultaneous evaluation of fibrosis and steatosis, as well as being easily accessible, inexpensive, non-irradiating, repeatable, and well-accepted by patients, we can assume that this multiparametric dynamic approach can be used to screen patients at risk. At the initial contact with the patient, after a few minutes of examination, a detailed multiparametric assessment of the liver characteristics, that contains information about liver structure, liver fibrosis, steatosis and inflammation can be obtained.

The future development of these multiparametric techniques and their implementation in clinical practice will provide a tremendous advantage not only to the hepatologists, but also to the primary care physicians and to any physician who performs abdominal ultrasound.

5. CONCLUSIONS

- (1) The new two-dimensional shear wave elastography technique (2D-SWE PLUS) with a new quality criterion (SI) was found to be a highly feasible method, with excellent performance in diagnosing and staging liver fibrosis.
- (2) THE proved to have excellent feasibility and reproducibility, with the highest performance in diagnosing liver cirrhosis.
- (3) SSp PLUS was found to be a highly feasible method, with good performance in detecting the presence of liver steatosis and in predicting significant steatosis.
- (4) Att PLUS showed excellent feasibility. However, SSp PLUS performed better than Att PLUS in the identification and staging of hepatic steatosis, demonstrating a stronger correlation with the control method.
- (5) Vi PLUS was found to be a highly feasible method. The mean liver viscosity measured by Vi PLUS in healthy subjects was 1.59 Pa·s.
- (6) Regardless of the underlying cause, the viscosity values obtained from CLD patients were significantly higher than those from the healthy cohort. Liver stiffness has a strong and significant effect on viscosity values.
- (7) The multiparametric ultrasound-based approach in NAFLD patients allows identifying and staging: liver stiffness using 2D-SWE PLUS; liver steatosis using Att PLUS and SSp PLUS; and liver viscosity using Vi PLUS.