

Comparison and correlation of fibrosis stage assessment by collagen proportionate area (CPA) and the ELF panel in patients with chronic liver disease

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ABSTRACT

Background. Fibrosis progression is the common consequence of most chronic liver diseases. **Aims.** To evaluate the performance of Collagen Proportionate Area (CPA) and ELF using Ishak's score in patients with chronic liver diseases.

Methods: Retrospective analysis of medical data from patients on whom a liver biopsy was performed as part of the diagnostic assessment. CPA was calculated by using digital image analysis and then compared with Ishak and ELF scores.

Results: 143 patients (84 men (59%); mean age 48.8 ± 12.8 years) were evaluated. Patients were mainly affected by viral hepatitis (92 HCV and 8 HBV). CPA and ELF values increased with worsening Ishak stage ($p < 0.001$) and their median values were significantly different among Ishak stages ($p < 0.001$). There was a significant correlation between CPA and ELF ($r = 0.5$). In AUROC analysis, CPA and ELF had similar diagnostic accuracy in identifying cirrhosis, but CPA had higher diagnostic accuracy than ELF in identifying significant or absent fibrosis. High ELF scores were observed in non-cirrhotic patients who suffered non-liver related deaths. **Conclusions:** This study demonstrated that

CPA and ELF values successfully identified patients with advanced fibrosis or cirrhosis. Moreover, ELF values successfully identified advanced fibrosis or cirrhosis, thus confirming the role of ELF as a clinical method for non-invasive assessment of fibrosis stage in chronic hepatitis.

INTRODUCTION

Fibrosis progression is the common consequence of most chronic liver diseases and histologic examination of a liver biopsy specimen is the reference standard for the assessment of liver fibrosis. However, liver biopsy is burdened by invasiveness and low degree of acceptance by patients, potentially lethal complications, sampling error, observer-dependent diagnostic variability and handling costs [1]. In the last decade, non-invasive and reproducible methodologies, with an acceptable level of diagnostic accuracy have been proposed for the non-invasive assessment of fibrosis [2,3]. These mainly include the measurement of tissue elasticity by transient elastography and serum markers of liver fibrosis [4,5]. The “indirect serum markers” are panels of clinical and biochemical parameters not directly related to extracellular matrix metabolism while the “direct serum markers”, such as the serum levels of molecules diffused into the systemic circulation, are related to the metabolism of the extracellular matrix [6]. Currently, the ELF score is derived by the assessment of direct serum markers of fibrosis, namely hyaluronic acid, aminoterminal propeptide of type III collagen and tissue inhibitor of matrix metalloproteinase 1 [7].

All non-invasive fibrosis tests to date were developed and calibrated with reference to semi-quantitative histological scoring systems, such as Ishak’s or METAVIR, rather than quantitative histological measurement of fibrosis, which would be more accurate and appropriate. Indeed, existing scoring systems do not represent a measurement of quantitative fibrosis, but rather a categorical description of both architecture and fibrosis. Collagen proportionate area (CPA)

is a validated method for the quantification of fibrosis by measuring hepatic collagen using digital image analysis [8-13]. This method was first described by Calvaruso et al. [8], who determined relationships between computer-assisted digital analysis, Ishak score and HVPG. The authors found a significant relationship between CPA and HVPG, indicating that computer-assisted digital image analysis measurement of CPA has clinical relevance, because it could be useful to stratify prognostic groups. Based on these premises, the aims of this study was to evaluate the diagnostic accuracy of CPA for the diagnosis of fibrosis stages using a standard semi-quantitative method (Ishak's score) and to provide a further validation of ELF by comparing ELF with a method (CPA) able to assess liver fibrosis quantitatively.

STUDY DESIGN AND METHODS

The study was performed on 143 liver biopsy samples of available tissue blocks largely from patients with HCV-related chronic liver disease, obtained at the time of the original ELF study at the University of Florence University Hospitals (AOUC) in Florence, Italy, as a part of an international, multicenter, cross-sectional cohort study [7].

In the original ELF study [7], patients were considered eligible if they were due to undergo liver biopsy for the investigation of chronic liver disease, defined as abnormal biochemical liver function tests persisting for more than 6 months, ability to provide informed consent and age between 18 and 75 years. Patients were excluded from the study if their age fell outside of this range; for any disorder associated with extrahepatic fibrosis, including rheumatic, renal, or lung disease; for cardiovascular disease or cancer; for advanced cirrhosis with evidence of decompensation (Child–Pugh class C); for consumption of regular aspirin; or for hepatocellular carcinoma or drug-induced liver disease.

Paraffin embedded tissue blocks were transferred from the University of Florence, Florence, Italy, to the Department of Pathology at the Royal Free Hospital, London, UK, where the study was performed. Each tissue block was processed according to the reported CPA method and analysed for collagen content. Results of the CPA analysis were compared with the standard Ishak's semiquantitative score [14] used in the original ELF study [7]. Due to possible differences in the cut of the paraffin blocks, i.e. progressive reduction of the block thickness, all samples were re-staged according to the Ishak's score. The

results of the CPA analysis were then compared with the ELF panel together with relevant clinical and biochemical parameters available in the patients' CRFs. All the above data were also re-analyzed by appropriate statistical methods and were fully reconsidered in order to find the best fit in defined mathematic cut-off.

CPA MEASUREMENTS

The quantitative measure of fibrotic tissue was calculated as the CPA using digital image analysis. The liver samples were formalin-fixed and paraffin-embedded for histological analysis. Sections of liver tissue were stained with haematoxylin/eosin and Sirius red. Only samples with a >25 mm length and including at least 11 complete portal tracts were considered adequate for the study, stained with Sirius red and used for calculating the percentage of collagen. The image was captured by a Canon Powershot A640 digital camera. The software used for the image analysis was Zeiss KS300 image analysis software. Measuring CPA involved capturing the whole image of a stained liver section at low-power magnification. Employing the Zeiss KS300 software, a greyscale slider was used to select the tissue area from which was calculated a total tissue area in pixels. This software enables, through a greyscale slider, to select the total tissue area of liver biopsy. Subsequently, red, green, and blue (RGB) light channels were used to select the collagen area. The percentage of total and collagen area was calculated. After whole-section digital image capture, the measurement included editing to eliminate structural collagen in large portal tracts, blood vessel walls, artefacts, vascular cavities, and lymphoid aggregates.

STATISTICAL ANALYSIS

All results are expressed as mean \pm standard deviation. The numerical comparison of continuous data was performed using the Student's t-test with Bonferroni correction, after checking similar variances in the groups by Levine's test for equality of variances. Correlation between variables was evaluated by Pearson's correlation coefficient (r). The Kruskal-Wallis one-way analysis of variance was used for comparing two or more independent samples.

Linear regression analysis was used to assess the relationship between ELF and CPA.

Considering CPA, we divided the range of values in each patient group into quartiles. The cut-offs at 25, 50 and 75 and > 75 % were compared for the four groups of patients, and the median ELF was calculated for each quartile.

The discriminative ability of CPA and ELF to predict fibrosis stages (significant fibrosis, advanced fibrosis and cirrhosis) according to the Ishak stage was assessed by receiver operating characteristic (ROC) analysis. The DeLong test was used to compare the AUROCs of CPA and ELF for specific stages of fibrosis.

The Cox regression method was used for assessing the predictive of CPA and ELF on liver-related events (varices, ascites, encephalopathy or HCC) and/or mortality.

Statistical significance was set at a value of $p < 0.05$. Statistical analysis and graphs were obtained using statistical software Stata 12, (College Station, TX, USA) and SPSS® v22 (IBM® Corporation, NY, USA) and the MedCalc software (Software for Windows, Version 14.8.1, Ostend, Belgium).

RESULTS

We evaluated 143 patients (84 men (59%); mean age 48.8 ± 12.8 years; range, 24-75). We excluded 28 biopsy specimens because of inadequate histological samples. Most patients were affected by viral hepatitis (92 HCV and 8 HBV), while 6 had NAFLD, 2 Primary Biliary Cholangitis and 7 various other etiologies. 28 (24.5%) patients had Ishak stage score of 0, 33 (29%) had Ishak stage score 1, 16 (14%) had Ishak stage score of 2, 14 (12%) patients had Ishak stage score of 3, 10 (9%) had Ishak stage score of 4, 9 (8%) patients had Ishak stage score of 5, 4 (3.5%) patients had Ishak stage score of 6.

The mean of CPA% and ELF score in the study population was 4.70 ± 4.46 (range: 0%-26%) and 9.40 ± 1.15 (range 6.98 - 12.99), respectively. The main clinical parameters of the study population are shown in Table 1.

RELATIONSHIP BETWEEN ISHAK SCORING, CPA AND ELF

The CPA values increased with worsening Ishak stage (Figure 1, Panel A). The median value of CPA measurements at each Ishak stage are shown in table 1. The Kruskal-Wallis one-way analysis of variance showed significant median differences between groups ($p < 0.001$). We found a similar relationship between ELF and Ishak stage score (Figure 1. Panel B).

The median values of ELF by Ishak stage are shown in table 1. The Kruskal-Wallis equality-of-populations rank test showed significant median differences between groups ($p < 0.001$).

Patients with advanced fibrosis (stages 4, 5, 6 stage), had significantly higher CPA and ELF values: $P < 0.001$; (3.3 versus 10.26 and 9.17 versus 10.36 respectively, $P < 0.0001$ for both comparisons, Figure 2).

Although CPA and ELF stratified by the severity of liver fibrosis did not correlate significantly (Figure S1), there was a significant correlation between CPA and ELF across all stages of liver fibrosis ($r = 0.5075$; $p < 0.001$) (Figure 3).

COMPARISON OF THE DIAGNOSTIC ACCURACY OF ELF AND CPA

We evaluated the area under the receiver operating curve (AUROC) for the CPA and ELF (Figure 4) in relation to 1) presence of cirrhosis (Ishak 0-4 vs 5-6): the AUROC were 0,890 (95% confidence interval (CI), 0,821 to 0,940) and 0,880 (95% CI, 0,821 to 0,932), respectively, $P = 0,8247$); presence of advanced fibrosis (Ishak 0-3 vs 4-6): the AUROC were 0,926 (95% CI, 0,865 to 0,966) for CPA and 0,785 for ELF (95% CI, 0,702 to 0,854), $P = 0,0147$; 3) presence

of significant fibrosis (Ishak 0-1 vs 2-6): the AUROC were 0,940 (95% CI, 0,882 to 0,975) for the CPA and 0,681 (95% CI, 0,591 to 0,763) for ELF, $P < 0,0001$; presence of any fibrosis (Ishak 0 vs 1-6): the AUROC were 0,879 (95% CI, 0,808 to 0,931) for the CPA and 0,719 (95% CI, 0,631 to 0,797) for ELF, $P < 0,0029$.

The diagnostic accuracy of CPA% and ELF in predicting all fibrosis stages of fibrosis is shown in table 2.

Considering each quartile of CPA we calculated the median value of ELF (table 3), for $CPA \geq 6\%$ the AUROC for ELF was 0.68 (95% CI 0.55-0.80) and the best cut-off was 10 (73.5% sensitivity and 57.1% specificity).

DESCRIPTION OF OUTCOMES

Patients had a mean follow-up of 7.94 ± 5.04 years (median=7 years). Follow-up ended in 2013 (12 years after the start of the study). We found a low number of events, probably due to the high number of patients with fibrosis stage 0-2 at baseline (77 patients). Moreover, 27 patients underwent antiviral treatment with interferon plus ribavirin (none of these underwent IFN-free therapy), of whom 17 (63% of those treated) reached sustained virological response. It is well established that antiviral therapy improves outcomes in HCV patients [15]. There were 9 events in total, which were as follows: 1 patient developed decompensated cirrhosis; 2 patients developed HCC; 1 patient

developed decompensated cirrhosis and hepatocellular carcinoma; a total of 5 patients died. In particular, 1 patient followed until 2013 developed encephalopathy in 2009, varices in 2010, and ascites in 2013 (this HCV patient had CPA%=2, Ishak score=G2,S0, and ELF=9.4 at baseline); one patient died in 2012 from hepatocellular carcinoma (this HCV patient had CPA%=5, Ishak score=G6,S3, and ELF=9.1 at baseline); one developed hepatocellular carcinoma in 2009 (this HCV patient had CPA%=16, Ishak score=G7,S6, and ELF=12.3 at baseline), and died in 2009; another one developed varices and hepatocellular carcinoma in 2005 (this HCV patient had CPA=23, Ishak score=G5,S6 and ELF=11.7). Out of five patients who died, the cause of death was non-liver related in three cases: 1 patient in 2002 (this HCV patient had CPA% 3, Ishak score=G5,S1, and ELF=9); 1 in 2009 (this patient had CPA%=2, Ishak score=G2,S2 and ELF=10.2 at baseline); 1 in 2005 (this HCV patient at baseline had CPA%=2, Ishak score=G5,S1, and ELF=9.5).

The cox regression analysis did not show a significant correlation of ELF (HR: 1.176, 95% CI: 0.694-1.991) or CPA (HR: 0.851, 95% CI: 0.557-1.300) with liver related outcomes and/or mortality.

DISCUSSION

In this study, a large cohort of patients was evaluated by both Ishak score and CPA, using digital image analysis. We quantified collagen and ELF scores in relation to each stage of the Ishak score. This study **established that** CPA and ELF values successfully identified patients with advanced fibrosis or cirrhosis. However, when we performed the AUROC analysis, CPA was superior to ELF for identifying patients with pre-cirrhotic stages of fibrosis.

Several studies [16-20] were conducted using non-invasive methods for studying and monitoring patients, which have largely replaced liver biopsy in clinical practice. As mentioned previously, existing scoring systems do not represent a measurement of quantitative fibrosis, but rather a categorical description of both architecture and fibrosis and, therefore, an imperfect “gold standard” for the quantitative measurement of liver fibrosis that requires the use of continuous variables. CPA is a morphometric collagen measurement that generates continuous variables that suffer less from the variance entailed in staining procedures and differing operator experience. Non-invasive markers of liver fibrosis should hence be validated using the CPA method.

The present study showed good detection of advanced fibrosis and cirrhosis in patients with chronic viral hepatitis. Recently, Fagan et al. [21] performed a non-invasive assessment of fibrosis severity by ELF, determining that the cut-off value of ≥ 9.8 identified advanced fibrosis with diagnostic accuracy, even if it is influenced by age, steatosis and histological activity. It was also the case in our study that an ELF score ≥ 10 was associated with advanced fibrosis (Ishak’s

stage \geq 4). In fact, the correlation between CPA and ELF is better for values up to 10 but is worse after that. ELF is a clinical method for the rapid and non-invasive assessment of fibrosis stage in chronic viral hepatitis, which is continuously subjected to extensive validation to exclude the factors influencing the fibrosis stage. Lichtinghagen et al. [22] measured ELF scores in 400 healthy controls and 79 CHC patients. Analysis of ELF scores in healthy subjects revealed that afternoon values were slightly higher than morning values, possibly as a result of food intake.

Even if the data concerning the outcomes were poor, it is evident that clinical events occur when the ELF score is ≥ 9 . Accordingly, Irvine et al. [23] showed that an ELF score indicative of advanced fibrosis (≥ 9.8) correlated with liver-related clinical outcomes. In our study, patients with concomitant extra-hepatic diseases that could potentially increase ELF levels were excluded. Abignano et al. [24], for instance, studied 210 patients with systemic sclerosis, demonstrating that ELF score, blindly, is a clinical-grade serum test that significantly correlates with fibrosis measurements in systemic sclerosis and with overall disease activity, severity, and health assessment questionnaire-disability index. Gonzalez-Lopez et al. [25], meanwhile, showed that procollagen type I and III aminoterminal propeptide correlated with interstitial lung disease patterns and severity in Mexican women with systemic sclerosis. A recent study by van der Voort et al. [26] of 531 patients applied existing cut-offs of ELF for patients and healthy controls and compared it with procollagen-3 N-terminal peptide (P3NP) test in patients with psoriasis, psoriatic arthritis and

rheumatoid arthritis, and controls. ELF score (> 11) and P3NP were found to be highest in patients with rheumatoid arthritis, followed by patients with psoriasis and psoriatic arthritis. Considering that ELF consists of hyaluronic acid, aminoterminal propeptide of type III collagen, and tissue inhibitor of matrix metalloproteinase 1, all pathologies that cause an increase in these markers are potentially able to determine an increase in ELF. In particular, serum hyaluronic acid level may be used as tools to differentiate between acute and chronic kidney injury [27]. Collagen metabolism was altered in cases of acute respiratory failure [28]. In this study, out of 5 patients with clinical outcomes, 3 patients (who had a non-liver related death) had an ELF level between 9.8 and 11.3 despite a low CPA and Ishak stage. This implies that ELF in this “gray area”, according to what observed Lichtinghagen et al. [22] cannot be clearly attributed to an advanced stage of fibrosis. It is plausible that such values could be a surrogate for non-liver related comorbidities that increase non-liver related morbidity and mortality, however this will need to be tested in larger cohorts. Therefore the potential utility of ELF in predicting non-liver related deaths should be further explored in larger prospective cohorts.

As for the majority of non-invasive methods, an ELF score does not suffice to accurately differentiate between the different stages of liver fibrosis; it may show if fibrosis is present or not or if overt liver cirrhosis is present, but it cannot differentiate between stages. Liver biopsy has long been considered the ‘gold standard’ for the evaluation of hepatic fibrosis and still remains the gold standard for proper diagnosis in some diseases.

Nevertheless, of the different types of scoring systems so far proposed, each of these [14, 29, 30] considered some aspects that could describe the fibrotic evolution, though none provided a quantitative assessment. Calvaruso et al. [8] showed that CPA had a better histological correlation with HVPG than Ishak stage. Similarly, Tsochatzis et al. [12] compared the performance of histological semi-quantitative and quantitative methods and found that CPA accurately subclassified cirrhosis; it is the only independent predictor of clinical decompensation among the other histological systems described to date. Furthermore, in our study, the AUROC analysis revealed CPA values as the best means to identify fibrosis stage.

The use of liver biopsy in selected cases is thus essential. Non-invasive fibrosis markers facilitate the initial screening of patients, so that liver biopsies are targeted to patients with high risk of significant or advanced fibrosis.

In conclusion, this study **demonstrated that CPA and ELF values successfully identified patients with advanced fibrosis or cirrhosis**, thus confirming the role of ELF as a clinical method for non-invasive assessment of advanced fibrosis stages in chronic hepatitis. CPA has superior diagnostic accuracy for lesser fibrosis stages.

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Fig. 1. Panel A. CPA values according to the Ishak stage score in 114 liver biopsies from patients with chronic liver disease. **Panel B.** ELF values according to the Ishak stage in 114 liver biopsies from patients with chronic liver disease.

Fig. 2. Collagen proportionate area and ELF values in 114 liver biopsy specimens from patients with chronic liver disease in the group 1 (Ishak stages 0-3) or in the group 2 (Ishak stages 4-6). The mean of CPA% and ELF values were statistically different.

Fig. 3. Correlation between CPA and ELF in 114 liver biopsies of patients with chronic liver disease.

Fig. 4. Receiver operating characteristic (ROC) curve showing the prediction of fibrosis with CPA% and ELF in the absence of fibrosis (Ishak 0-1 vs 2-6) (Panel A), in moderate (Ishak 0-1 vs 2-6) (Panel B), severe fibrosis (Ishak 0-3 vs 4-6) (Panel C) and cirrhosis (Ishak 0-4 vs 5-6) (Panel D). The ideal area under the curve (AUC) is 1.00. The straight line represents that based on chance alone (AUC=0.50).

Fig. S1. CPA and ELF stratified by the severity of liver fibrosis. Correlation between CPA% and ELF in the absence of fibrosis (N=28; Panel A), in the presence of fibrosis corresponding to Ishak score of 1 (N=28; Panel B), Ishak score of 2 (N=33; Panel C), Ishak score of 3 (N=15; Panel D), Ishak score of 4 (N=14; Panel E), Ishak score of 5 (N=10; Panel F) and cirrhosis (N=9; Panel G).