Full Title:

NON-INVASIVE MARKERS OF LIVER FIBROSIS: ON TREATMENT CHANGES OF SERUM MARKERS PREDICT THE OUTCOME OF ANTI-FIBROTIC THERAPY

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ST, PT, BH, JP, EN, MO, KW, CH, DN, DS and WMR contributed to the study design and manuscript drafting. EE, PG, EN, MO, KW, CH, DS were responsible for patient recruitment, sample collection and processing. DN and OD scored the liver biopsy specimens used in the study. ST, JP, and SH performed the statistical analyses.

Conflicts of Interest: WR and DS are inventors of the ELF test. WR has received support for research studies and speaking events from Siemens Healthcare Diagnostics Inc. All the other authors have no conflicts of interest.

Abbreviations: AUROC, area under receiver operating characteristic curve; ELF, Enhanced Liver Fibrosis; HA, hyularonic acid; CHC, chronic hepatitis C; PEG-INFα2b, pegylated interferon alfa-2b; PIIINP, terminal peptide of pro-collagen III; TIMP-1, tissue inhibitor of matrix metaloproteinase-1; SVR, sustained virological response; SD, standard deviation; NS, non-significant at 0.05 threshold; ordAUROC, unweighted Obuchowski measure

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ABSTRACT

Background & Aims: the utility of non-invasive serum markers to longitudinally monitor liver fibrosis is not established.

Methods: this study included 70 patients with chronic hepatitis C who having previously failed interferon-based antiviral therapy, were randomized to receive pegylated interferon with or without silymarin for 24 months. ELF tests (HA, PIIINP, TIMP-1) were performed on patient sera taken prior to, during and at the end of the study (0, 12, 24 months) and liver histology obtained prior to and at the end of the study.

Results: following the study, absolute changes in Ishak fibrosis stage and ELF ranged from -4 to +4 and -2.41 to +2.68, respectively. Absolute changes in ELF at study mid-point were significantly associated with changes in both ELF and histology at the end of the study. A model combining both baseline ELF and change of ELF at study mid-point was able to predict the end of study ELF *(R^2=0.609, p-value<1x10^{-11})*, a decrease in ELF *(AUC:0.80-0.85)*, and a rise in ELF *(AUC:0.81-0.85)*. Furthermore, a model combining both baseline histologic stage and ELF together with the change of ELF at study mid-point was able to predict end of study histology *(R^2=0.601, P-value<1x10^{-11}, AUC:0.88-0.92)*, histologic fibrosis regression *(AUC:0.81-0.84)* and progression *(AUC:0.86-0.91).*

Conclusions: our observations suggest that a change in the non-invasive serum marker ELF predicts changes in liver fibrosis over a longer period. These data support the use of ELF as a surrogate marker of liver fibrosis evolution in monitoring anti-fibrotic treatments thus permitting “response-guided” therapy by the early identification of patients who will benefit from prolonged anti-fibrotic treatment.

INTRODUCTION

Progression of chronic liver diseases including chronic hepatitis C (CHC) to cirrhosis is increasingly recognized as highly important, if not the most important clinical endpoint, since cirrhosis incurs a high risk of portal hypertension, liver failure and hepatocellular carcinoma.[1, 2] The histological staging of a liver biopsy remains the reference standard for assessing hepatic fibrosis. However, reliability of liver biopsy is limited due to sampling error [3], inter- and intra-observer variability[4], and procedural complications.[5] As a result, several non-invasive methods have been developed for the cross-sectional staging of liver fibrosis. These include both direct (ELF[6], Fibrospect[7]) and indirect (APRI[8], Fibrotest[9], Hepascore[10], Fibrometer[11]) serum markers and imaging techniques (Fibroscan[12], ARFI[13], MR elastography).[14] **Although biological plausibility links direct serum markers of fibrosis to either fibrolytic or fibrogenic processes involved in liver matrix turnover[15] it must be emphasized that direct markers are neither completely liver or fibrosis specific.**

Compared to liver histology, non-invasive methods have demonstrated robust performance in the detection of moderate or advanced hepatic fibrosis in a variety of chronic liver diseases. **Although there is a now a wealth of data supporting the use of biomarkers to track histologic fibrosis longitudinally during the natural history of a patient's liver disease what has been less reliably demonstrated is their ability to monitor fibrosis during anti-fibrotic therapy.**

This study was based on the PROFI-C (Progression of Fibrosis Inhibition in Hepatitis C) randomized trial which investigated whether previous non-responders or relapsers to interferon based therapy showed delayed fibrosis development after 24

months of treatment with a combination of interferon alpha and either silymarin or placebo. Patients in the PROFI-C study had serum samples taken prior to, at the mid-point and at the end of therapy. The scientific rationale for the study was based upon previous work where both interferon alpha and silymarin were studied as putative anti-fibrotic agents. Earlier studies suggested that the administration of interferon alpha to patients with CHC was associated with significant histological improvement[16, 17] and silymarin demonstrated a marked anti-fibrotic effect in rodent models of hepatic fibrosis.[18-20]

Whilst the PROFI-C study itself did not demonstrate an appreciable difference in histological outcomes between the treatment arms[21], the serum and histological samples taken during the trial have provided an invaluable platform to evaluate longitudinal changes of liver fibrosis as assessed by both non-invasive serum markers and liver histology. Using this cohort, we explored, whether changes in liver fibrosis assessed by both liver histology and serum markers at the end of the study period are associated with baseline and on-treatment changes in serum markers.

METHODS

Study Design

Patients in this study were enrolled in the PROFI-C trial. Written informed consent was obtained from all patients before admission to the study. Ethical approval was granted by the local ethics committees of the participating centers in accordance with the guidelines of the 1975 Declaration of Helsinki. PROFI-C was an investigator-initiated, prospective, randomized trial involving 18 centers in Germany and Austria (supplementary data), investigating the effect of high dose silymarin plus pegylated interferon alpha 2b (PEG-INFα2b, PegIntron, Essex Pharma GmbH, Munich, Germany) in non-responders or relapsers to standard treatment for CHC. Whereas 108 patients were enrolled into the PROFI-C trial[21], only patients who underwent consecutive liver biopsy (prior to and after the 24 month therapy) and had stored sera taken prior to, at the mid-point, and at the end of therapy (0, 12, 24 months) were evaluated in this study (n=70). **All participants were fasting at the time of serum sampling.** Participants were male or female patients aged between 18 and 65 years with chronic hepatitis C infection and had evidence of CHC (positive tests for anti-HCV antibodies and HCV-RNA (COBAS Amplicor HCV Monitor, Roche Molecular Diagnostics, Mannheim, Germany) after failure of first-line therapy with either interferon or pegylated interferon and ribavirin. Patients were also required to have histologically proven chronic hepatitis on a liver biopsy specimen (at least 8 identifiable portal tracts) within 6 months prior to entry into the study.

Exclusion criteria included treatment with silymarin, steroids or immunosuppressive drugs in the preceding three months, acute hepatitis, Child-Pugh stage B or C cirrhosis, thrombocytopaenia ($<100 \times 10^9$/L), leucopenia ($<3 \times 10^9$/L), other chronic

liver diseases, history of liver or kidney transplantation, autoimmune diseases, HIV infection, active hepatitis B infection, alcohol abuse (defined as the consumption of >40g per day in males and 20g per day in females) in the preceding 6 months, active drug abuse, pregnancy and lactation, severe somatic (renal, cardiac, pulmonary, gastrointestinal, oncologic) or psychiatric diseases and depression.

Randomization and data collection were performed at the Department of Medicine, University Hospital Erlangen, and all virologic and serologic analyses were performed according to standardized laboratory routines.

Treatment Schedule

All patients were treated with subcutaneous PEG-INFα2b at either 100 µg per week or at 50 µg on alternate weeks. Treatment was combined with oral silymarin (Bionorica Arzneimittel, Neumarkt, Germany) treatment at 280 mg three times per day (280mg per capsule) or an identically encapsulated placebo filled with glucose and soy bean extract in one of the 4 following treatment regimens:

1) PEG-INFα2b (100 µg/week) + silymarin

2) PEG-INFα2b (100 µg/week) + placebo

3) PEG-INFα2b (50 µg/every other week) + silymarin

4) PEG-INFα2b (50 µg/every other week) + placebo

The treatment period was 24 months with an additional 3 months of post-treatment surveillance. During the therapy, patients were evaluated 3 monthly to monitor for side effects, compliance and changes in hematological, biochemical and virologic parameters.

Histological assessment

Liver histology was obtained at the beginning of the study and at the end of the treatment period (month 24 ± 3 months). Biopsies were fixed in formalin and embedded in paraffin. Hematoxylin-Eosin staining was used for grading of inflammation and the Chromotrope-aniline blue staining for staging the amount of liver fibrosis. [22, 23] All specimens were graded and staged by using the Ishak score.[24] Histological assessment was performed by 2 independent pathologists (D.N. and O.D.) who were blinded to the clinical data and randomization status of the patients in the study. Interobserver variability was determined by the Kappa statistic (Kappa=0.624). All liver biopsy specimens that were discordantly staged were re-reviewed by both pathologists with a final score determined after further discussion.

Sample Collection and Serum Marker testing

Sera were stored at -70ºC prior to transfer to the central laboratory, where ELF tests were performed on thawed samples. Serum samples were analyzed for levels of TIMP-1, HA and PIIINP using the proprietary assays developed for the ELF test by Siemens Healthcare Diagnostics Inc (Tarrytown, New York, USA). These assays are magnetic particle separation immunoassays and were performed on the ADVIA Centaur® immunoassay system (Siemens Medical Solutions Diagnostics Inc, Tarrytown, New York, USA). Results were entered in to the manufacturer's published algorithms appropriate for the analyzer used to test the samples and to derive an ELF score.

Virologic Analysis

Hepatitis C virus (HCV) RNA was quantified using an in-house HCV RNA real time RT-qPCR [25] using the QIAamp96 Virus nucleic acid purification procedure on the BioRobotMDx (Qiagen, Hilden, Germany) and the ABI Prism 7500 real-time PCR with Qiagen QuantiTect probe RT-PCR reagents. The assay uses brome mosaic virus RNA as an internal control, introduced at the extraction stage.

HCV genotyping was performed by amplifying and sequencing a region of the 5'NCR. The sequence was analyzed to compare probe binding sites of the LiPa method [26] and by finding the restriction sites.[27] These two virtual methods were compared to give the HCV genotype result.

Statistical Analyses

Statistical analyses were performed using SPSS for Windows (version 20, SPSS Inc, Chicago, IL) and R for Windows (version 2.15.1, the R Foundation for Statistical Computing). Patient demographic and clinical laboratory characteristics were descriptively summarized and reported as mean ± standard deviation (SD) and range. All tests were two-sided and statistical significance assessed at the 0.05 threshold. The diagnostic performance of ELF as compared to liver biopsy was assessed using receiver operating characteristic (ROC) curves. The area under the receiver operating curves (AUROC) and 95% confidence intervals of AUROC were calculated. The Obuchowski[28] method of correcting for spectrum effect was applied in a similar fashion to previously published literature.[29] The Obuchowski measure (ordROC) gives a weighted average of the N(N-1)/2 AUROC pairwise comparisons

between N categories of gold standard outcome. Thus using the Ishak scale with its N (=7) categories of fibrosis staging (F0-6) there are 21 pairwise comparisons between 2 of the N categories. Each pairwise comparison can be weighted to account for the distance between fibrosis stages. Accordingly we defined a penalty function proportional to the difference in Ishak units between fibrosis stages. The penalty function was 0.17, 0.33, 0.5, 0.67, 0.83 and 1 when the difference between Ishak stages was 1, 2, 3, 4, 5 and 6 stages respectively. As the severity of histological liver fibrosis in patients with CHC with prior treatment failure has not been well characterized, the Obuchowski measure presented in this study has not been weighted according to the prevalence of fibrosis stages in a reference population. A 2-sided t-test was used to assess changes of mean Ishak biopsy and serum marker scores as parametric variables arising during the study period. Univariate correlation coefficients (Spearman's Rho) were calculated to assess the association between changes in serum marker scores and liver histology occurring at the end of the study period with baseline serum markers and changes in serum markers occurring at the mid-point of the study period. Linear and logistic regression were used to construct models incorporating baseline and on-treatment variables that were predictive of continuous and categorical variables respectively. The clinical utility of these models for predicting fibrosis progression and regression at the end of the study period was assessed using AUROC analysis. Sensitivity, specificity and predictive values were calculated at thresholds derived from ROC curves.

RESULTS

Patient characteristics and baseline histology

The baseline characteristics of the 70 patients included in this study are displayed in table 1. The participants in this study were comprised of patients who had been randomized into each of the 4 arms of the PROFI-C trial. Patients were mostly male and had a mean age of 48.5 years, with predominantly CHC genotype 1. Baseline hematological and biochemical parameters were compatible with compensated chronic liver disease.

The distribution of mean Ishak fibrosis score and mean ELF score prior to therapy are displayed in table 2. All 7 Ishak stages are represented with 26% of patients having severe fibrosis/cirrhosis (F5-6). The mean Ishak fibrosis stage prior to therapy was 2.9.

Effect of therapy on HCV RNA

The mean change in HCV RNA at month 12 was -0.78 log (range -7.20 to 2.14). Suppression of HCV RNA was more marked in those patients receiving PEG-IFNα2b 100μg (*p=0.008*). Seven patients achieved full suppression of HCV RNA during therapy of which 5 had been randomized to the PEG-IFNα2b 100 μg group. No patients achieved a sustained virological response (SVR). The addition of silymarin did not influence outcome (*p-value>0.05, non significant (NS)*).

ELF performs well at discriminating between fibrosis stages both at baseline and after putative anti-fibrotic therapy

The ELF test exhibited good performance in the detection of histological fibrosis at baseline and at the end of the study period. The performance of ELF in detecting

severe fibrosis or cirrhosis was similar (AUC 0.87-0.88) with the Obuchowski measure (ordAUROC) comparable both at baseline and at the end of the study period (ordAUROC 0.93-0.92, standard error 0.02).

A heterogeneous effect on both serum markers and histology is observed after putative anti-fibrotic therapy

Absolute changes in both histology and ELF score observed during the study are presented graphically in figure 1. At the end of treatment, absolute changes in Ishak stage and ELF score ranged from -4 to +4 and -2.41 to +2.68 respectively. Twenty one and 24 patients were noted to have an absolute decrease and increase in Ishak fibrosis score at the end of the study period, respectively (table 2 and figure 1). Similarly, 27 and 43 patients were noted to have an absolute decrease and increase in ELF score at the end of the study period respectively.

The dosage of pegylated interferon and the addition of silymarin did not influence anti-fibrotic outcomes *(p-value=NS).* In addition, the degree of viral suppression on therapy also did not influence outcome *(p-value=NS).*

Baseline and on treatment levels of ELF scores are associated with the evolution of ELF scores at the end of putative anti-fibrotic therapy

After stratifying patients by their end of treatment changes in ELF score (figure 2A), it was evident that mean baseline ELF scores were significantly higher *(p-value=0.043)* in patients who experienced a decrease in ELF score at the end of study than in those who experienced an increase in ELF score at the end of the study. Stepwise logistic regression identified the baseline ELF score as the only baseline factor associated with an end of study reduction in serum ELF *(OR 1.59, 95% CI 1.01-2.52,*

p-value=0.049). With regard to the marker scores during the treatment period, absolute changes in ELF score between months 0 and 12 were found to correlate with absolute changes between months 0 and 24 *(r=0.599, p-value <10^{-7})* suggesting that changes of ELF score that occurred between the start and the mid-point of the study were predictive of ELF scores at the end of the study. Moreover, mean ELF scores were observed to be significantly higher at the mid-point of the trial than they were at the beginning of the study (table 3) with the magnitude of this rise reflecting the outcome of the changes in ELF at the end of the study (Figure 2A).

Changes in serum markers observed during putative anti-fibrotic therapy reflect histological outcomes at the end of therapy

Patients were divided into 3 categories based upon the histologic outcome determined at the end of the study period. Histological fibrosis regression or progression was defined as ≥1 stage decrease or increase in Ishak fibrosis stage respectively. Within these 3 categories, mean changes of ELF scores were evaluated. These data are presented graphically in figure 2B. During therapy there was a rise in mean ELF score in all 3 categories of fibrosis evolution with the magnitude of this rise reflecting the change in histology observed at the end of the study. Patients with fibrosis progression had the largest rise in ELF followed by those with unchanged histology; those with fibrosis regression had the smallest change. Analogous to the pattern observed in the evolution of serum markers at the end of therapy, this effect was not sustained at the end of the study period with mean ELF scores at the end being lower than those seen at the mid-point of the study period. This pattern of rise and subsequent fall of serum markers was observed in patients receiving both dose schedules of pegylated interferon and in patients receiving either silymarin or placebo.

Changes in ELF score at the mid-point of the study correlate with changes in Ishak fibrosis score at the end of the study

The relationship between the absolute change in liver histological fibrosis (seen at the end of the study) and the absolute change in serum marker score (at the mid-point and end of the study period) was explored. Individual changes in ELF score observed at the mid-point of the study period significantly correlated *(p=0.007)* with individual changes of histological fibrosis score seen at the end of the study period. However, a significant association was not observed between the change in ELF score occurring prior to, and at the end of the study period with the change in Ishak fibrosis score occurring at the end of the study. The lack of a significant association between changes in serum markers at the end of the study and histological evolution at the end of the study may be explained by the observation that the end of study ELF scores were seen to regress thus reducing the power of the study to detect a significant association at this time point.

An 'ELF regression model' comprising baseline ELF and changes of ELF at the mid-point of the study can predict the both the ELF score and change of ELF score at the end of the study

Both baseline ELF score *(r=-0.249, p-value=0.038)* and changes in ELF score arising at the mid-point of therapy *(r=0.599, p-value<10^{-7})* were significantly associated with the change of ELF score at the end of the study period. An 'ELF regression model' comprised of both baseline ELF score and the change of ELF score at month 12 were able to predict the change of ELF score at month 24 *(R=0.626, R^2=0.392, p-value<10^{-8})* and the end of study ELF score *(R=0.781,*

R^2=0.609, p-value<10^{-11}) [figure 3A]. The change of ELF score at the end of the study is described by the equation: -5.786 - 0.762(ELF_0) + 0.665(ΔELF_{0-12}).

Performance of the 'ELF regression model' in predicting change in ELF

The performance of the 'ELF regression model' in predicting a change of ELF at the end of the study is presented in table 4. The performance of the regression model ranged from 0.80 to 0.85 and 0.81 to 0.85 in its ability to predict a fall and rise in ELF score respectively.

A 'histologic regression model' comprising baseline histology and on treatment changes of serum markers can predict the end of therapy histology and histologic change

Stepwise linear regression incorporating baseline serum marker scores was used to develop models able to predict histological outcome as assessed by a change in the semi-continuous Ishak stage arising at the end of the study period. Models incorporating both the baseline and subsequent changes in both ELF score and its constituent components did not result in a significant improvement in the univariate correlation coefficients already identified. However, the incorporation of baseline histology produced models with significantly improved performance. The best performing 'histologic regression model' combined baseline Ishak fibrosis score, baseline ELF score together with the change of ELF score arising at the mid-point of therapy *(R= 0.645, R^2 0.416, P-value = 9.2 x 10^{-8})* (figure 3B). The 'histologic regression model' score is described by the equation: -5.786 - 0.601($Ishak_0$) + 0.762(ELF_0) + 0.665(ΔELF_{0-12}). Similarly, the end of therapy Ishak fibrosis stage was best predicted by a model combining baseline Ishak fibrosis score, baseline ELF

score together with the change of ELF score arising at the mid-point of therapy *($R=0.775$, $R^2=0.601$, $P\text{-value}<1 \times 10^{-13}$).*

Performance of the 'histologic regression model' in predicting histologic change and end of treatment histology

The performance of the 'histologic regression model' in predicting histologic fibrosis evolution and the histologic fibrosis stage at the end of the study period was evaluated (table 5). The AUC for predicting fibrosis regression of greater than 1, 2 and 3 Ishak stages at the end of the study period was 0.81, 0.84 and 0.83 respectively. The AUC for predicting fibrosis progression of greater than 1, 2 and 3 Ishak stages at the end of the study period was 0.86, 0.91 and 0.88 respectively. Furthermore, the 'histologic regression model' performed well in its ability to discriminate between the end of study fibrosis stages (AUC 0.88-0.92, table 4).

Examples of how the 'histologic regression model' can be used predict fibrosis change at the end of therapy in clinical practice

A 'histologic regression model' threshold of +0.53 could be used to 'rule in' failure of anti-fibrotic therapy (a rise of more than 2 Ishak fibrosis stage at the end of the study period, sensitivity of 88%, specificity of 83%, diagnostic odds ratio of 34.2 and positive likelihood ratio 5.1) and 'rule out' successful anti-fibrotic therapy (a fall of more than 2 Ishak stages at the end of the study period ,sensitivity 100%, specificity 40%, negative likelihood ratio 0.0). As a result, patients with a 'histologic' threshold of greater than 0.53 at the midpoint of therapy could be considered unlikely to achieve significant fibrosis regression and could therefore stop therapy early by meeting a 'futility rule'.

Conversely, a ‘histologic regression model’ threshold of -0.63 could be used to ‘rule out’ failure of anti-fibrotic therapy (a rise of more than 2 Ishak fibrosis stage at the end of the study period, sensitivity of 100%, specificity of 30%, negative likelihood ratio 0.0) and ‘rule in’ successful anti-fibrotic therapy (a fall of more than 2 Ishak stages at the end of the study period, sensitivity 64%, positive likelihood ratio 4.3, diagnostic odds ratio 10.1). A ‘histologic threshold’ of less than -0.63 at the midpoint of therapy could be applied as a ‘continuation rule’ with patients scoring less than -0.63 considered likely to achieve fibrosis regression.

DISCUSSION

Hitherto, it has been well documented that fibrosis biomarkers or imaging techniques are able to longitudinally monitor liver fibrosis during treatment for chronic viral hepatitis.[30] Studies have compared histological change occurring after putative anti-fibrotic therapy with change in serum markers seen at the end of therapy but not during therapy [31-33] and have also monitored changes in serum markers occurring during putative anti-fibrotic therapy but have not had sequential histology as an end point. [34-38] **However,** to our knowledge, this is the first study that has evaluated changes in serum markers occurring during a clinical trial with changes of fibrosis stage at the end of the trial period. As a result, this **proof of concept** study provides a valuable insight into the dynamic interaction and association between changes in fibrosis and the levels of a panel of serum markers which most plausibly may reflect the dynamics of liver fibrogenesis and fibrolysis. [39] As a result, the strength of our study lies in the ability to explore the association between the evolution of histology between baseline and the end of therapy with the dynamic evolution of serum marker scores measured at baseline, study mid-point and the end of therapy.

In this study, we have demonstrated that patients with lower pre-treatment ELF scores have demonstrated greater increases in ELF score at the end of the study period. In addition, we have observed that changes of ELF occurring at the mid-point of the study period are significantly associated with fibrosis evolution at the end of the study.

The ‘ELF regression model’ which combined these two observations (baseline ELF score and a change of ELF score at the mid-point of the study) has performed well at

identifying improvement in fibrosis as defined by a fall in ELF score at the end of therapy (AUC 0.80-0.85), and worsening of fibrosis as defined by a rise in ELF score at the end of therapy (AUC 0.81-0.85). Furthermore, the 'histologic regression model' which combined these two observations with baseline histologic fibrosis stage performed well at identifying improved fibrosis (AUC 0.81-0.84) and worsened fibrosis (AUC 0.86-0.91) as assessed by histological change.

The purely non-invasive 'ELF regression model' combining only baseline and changes of ELF score at the mid-point of therapy performed less well at predicting histological fibrosis evolution. For the prediction of histologic fibrosis regression the performance of this model ranged from AUC of 0.64 for predicting the regression of at least 1 Ishak stage to AUC of 0.75 predicting the regression of at least 3 Ishak stages. However, for the prediction of histologic fibrosis progression, the performance of this 'non-invasive' model was more comparable to the model incorporating baseline histological stage with AUC of 0.72 for predicting the progression of at least 1 Ishak stage to AUC of 0.85 for predicting the progression of at least 3 Ishak stages. Regardless, the non-invasive 'ELF regression model' (based on baseline and on treatment changes of ELF) performed well in its ability to discriminate between fibrosis stages at the end of the study (AUC 0.83-0.91).

Whilst the sample size in this study is modest, the challenge of obtaining paired liver biopsies from patients with serial blood samples over 24 months is increasingly difficult, in part due to recognition of the accuracy of non-invasive methods for liver fibrosis staging. Our study population represents a subgroup of patients enrolled in a randomized controlled trial that attempted to investigate the anti-fibrotic properties of interferon and or silymarin. The trial found that none of the therapeutic regimes were superior to any of the others studied in delivering a significant benefit in terms of liver

fibrosis. Due to the lack of a control arm (in which patients did not receive therapy) the study did not permit exploration of the correlation between any potential anti-fibrotic effect attributable to interferon based therapy. This however has already been addressed by larger randomized controlled trials which have collectively suggested that interferon is not superior to placebo in preventing histological progression when sustained viral response is not achieved. [40, 41] Nevertheless, in this study, two independent methods of assessing liver fibrosis, non-invasive serum markers and liver histology, have documented significant changes in liver fibrosis. Up to 70% of the patients in the study experienced either no progression or regression of fibrosis as assessed by either methodology suggesting that the treatment may have had some anti-fibrotic effect. However, our histologic findings are similar to a previous study of 219 untreated patients with CHC who had interval biopsies after a median interval of 2.5 years.[42] In this study 33% and 10% of patients showed progression and regression of liver fibrosis by at least 1 Ishak stage, respectively.

Regardless, when considering a single modality to assess liver fibrosis, changes in either histology or serum markers at the end of the study period could be attributed to confounding factors such as sampling error. **This may seem plausible given that some patients were noted to have an change of up to 4 Ishak points following therapy. However**, within our study we have observed that changes in these two independent modalities of assessing liver fibrosis are significantly associated with one another. Thus, the correlation between changes in ELF and changes in histology is likely to be attributable to a true biological association between these two methods of assessing liver fibrosis rather than the result of confounding or a random association. The correlation of change in ELF with improvement of histological fibrosis and the corresponding correlation of increase in ELF with progression of

fibrosis provides evidence that the ELF test can be used to assess longitudinal changes of fibrosis. This could be in the context of therapeutic trials of drugs that lead to improvements in liver fibrosis either as a result of successful treatment of underlying pathology (such as clearance of HCV or HBV infection) or due to a direct anti-fibrotic effect, as was anticipated in the PROFI-C trial. Furthermore, the ability to predict longer term changes in fibrosis by the use of on-treatment changes in serum markers would enable clinicians to employ ‘futility rules’ for such therapies by the use of clinically relevant thresholds. Patients meeting ‘futility rules’ based upon relevant diagnostic thresholds would be able to discontinue therapy early thus avoiding unnecessary exposure to agents.

Regardless, due to the introduction of directly-acting antiviral agents (DAAs) for the treatment of CHC the concept of maintenance interferon has now been surpassed by treatment regimens that offer SVR for the overwhelming majority of patients with increasingly short treatment durations. However, it is clear that hepatic fibrosis may evolve during a course of antiviral therapy the duration of which at present continues to be for up to 48 weeks for many patients. As a result, one would anticipate that fibrosis evolution occurring as the result of antiviral therapy is a relevant clinical consideration. In our study we have demonstrated that the performance of serum markers in detecting fibrosis remains consistent after a course of interferon based therapy and on treatment changes can be used to predict fibrosis evolution occurring as a result of therapy. Thus patients can be stratified into those who have and have not developed severe fibrosis or cirrhosis and screening for hepatoma and endoscopic features of portal hypertension can be instigated. Furthermore, it is anticipated that improvements in hepatic fibrosis will result in improved clinical outcomes in patients with advanced liver disease. Primarily, a reduction in ELF score

suggests an improvement in hepatic fibrosis. However, as ELF scores have been shown to predict clinical outcomes more reliably than histology[43], a rise or fall in ELF score resulting from therapy appears to denote an increase or decrease in the risk of subsequent liver related outcomes. **Conversely, changes in ELF score may also be representative of changes in hepatic inflammation. Our data demonstrate that improvements in liver fibrosis stage are accompanied by improvements in inflammation which potentially could overestimate of the anti-fibrotic effect of a treatment. However, within our data it is evident that the evolution of inflammation mirrors the evolution of fibrosis suggesting that changes in either histological parameter are unlikely to be witnessed alone. Furthermore, in patients with either an increase or decrease in liver inflammation a rise in ELF score was identified at the mid-point of therapy despite a concurrent fall in ALT.**

Whether or not our observations are restricted to interferon alpha or silymarin based treatment or are applicable to any anti-fibrotic therapy in general remains to be tested. Interestingly, our observations were consistent regardless of whether or not patients received pegylated interferon with or without silymarin in our study. We have noted a rise in ELF score during interferon therapy regardless of the evolution of fibrosis at the end of therapy. **Our data suggest that this is predominantly due to a rise in HA and PIIINP.** This has been previously noted by other investigators and has been attributed to the effect of interferon on extrahepatic serum markers of fibrosis.[36] However, the lower pegylated interferon dosage of 50 mcg at alternate weeks is indeed considerably lower than that normally employed for the treatment of CHC. It is therefore unclear whether the changes in serum markers that we have

observed in our study are due to interferon itself but instead due to the activation of fibrogenic pathways which result in a rise in serum markers.

Given that ELF scores have been noted to rise after the ingestion of food[44], it is important that the sera used to assess a change in ELF score are taken with patients in a fasting state. The sera itself should be allowed to clot for 30 minutes at room temperature after which centrifuging is performed; the use of frozen sera has not been shown to impact on Elf scores. [44]

In summary, our study has demonstrated that baseline and on-treatment changes of the ELF score are significantly associated with fibrosis evolution during a 24 month period of observation. A model combining these parameters was highly predictive of changes in ELF score over a longer period. In addition, when combined in a model with baseline histology these parameters were highly predictive of histologic fibrosis evolution over a longer period. If confirmed in other cohorts using anti-fibrotic agents, this may qualify ELF as a dynamic marker panel of fibrosis evolution rather than simply a parameter of cross-sectional fibrosis staging; this will be highly relevant for the clinical testing of a large number of upcoming anti-fibrotic agents.[45] It would also permit the refinement of “response-guided therapy” by identifying those patients who will benefit from both continued and prolonged anti-fibrotic treatment.

Figure Legends:

Figure 1: Histograms displaying the absolute changes of Ishak Fibrosis Stage between 0-24 months (A), and of the ELF score between 0-12 months (B) and 0-24 months (C) of the study period.

Figure 2: Changes in mean ELF during the study period after stratifying patients by the evolution of ELF (A), and by histologic fibrosis evolution (B) at the end of the study.

Figure 3: models predicting fibrosis evolution at the end of the study period. Figure 3A: a model combining baseline ELF and change of ELF at the mid-point of the study predicts the end of study ELF. Figure 3B: a model combining baseline Ishak stage with baseline ELF and change of ELF at the mid-point of the study predicts the end of study change of Ishak fibrosis stage.

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