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# Decreased IP-10 and elevated TGF$\beta$1 levels are associated with viral clearance following therapy in patients with hepatitis C virus

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**Abstract**. The role of pro-fibrogenic cytokines in the outcome of infections with hepatitis C virus (HCV) and the response to treatment with pegylated interferon-alpha (pegIFN$\alpha$) and ribavirin remains unclear. To address this issue, we assessed hepatic fibrosis and plasma markers pertinent to T-cell mediated fibrogenesis and inflammation at the start of treatment. Levels of soluble (s)CD30, interleukin-13 receptor alpha 2 (IL-13R$\alpha$2), total and active transforming growth factor-beta 1 (TGF$\beta$1), interleukin-18 (IL-18) and interferon-gamma inducible protein-10 (IP-10, CXCL10) were correlated with the severity of fibrosis and with treatment outcome using multiple logistic regression modelling. The Hepascore algorithm was confirmed as a marker of fibrosis, but was a poor predictor of treatment outcome. Inclusion of all immunological markers improved prediction based on Hepascore alone ($p = 0.045$), but optimal prediction was achieved with an algorithm ("TIPscore") based on TGF$\beta$1 (total), IP-10, age, sex and HCV genotype ($p = 0.003$ relative to Hepascore). Whilst this was only marginally more effective than predictions based on HCV genotype age and sex ($p = 0.07$), it associates high TGF$\beta$1 and low IP-10 levels with a failure of therapy.

Keywords: Hepatitis C virus, interferon-based therapy, chemokines, fibrosis

## 1. Introduction

The incidence and prevalence of HCV infections remains high worldwide. Primary HCV infection resolves in ∼20% of individuals, whilst most develop chronic infections that can persist for decades if untreated. Over a 10–20 year period, ∼20% of patients with chronic HCV progress to cirrhosis [3].

HCV infection is treated with pegylated interferon-$\alpha$ and ribavirin (pegIFN$\alpha$/RBV). Factors associated with a favourable treatment outcome include younger age, low pre-treatment HCV viral load, minimal liver damage and HCV genotype 2 or 3 [10]. Histological assessment of a liver biopsy has been the "gold standard" to demonstrate fibrosis, but carries a risk of complications [24]. Blood tests developed to assess fibrosis include FibroTest [11], Forn's score [6] and FIBROSpect [23]. The Hepascore algorithm uses age, sex and levels of bilirubin, $\gamma$-glutamyltransferase, hyaluronic acid (HA) and $\alpha_2$-macroglobulin to monitor fibrosis [1]. Comparison of non-invasive fibrosis mod-

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els demonstrated similar degrees of accuracy for detecting significant fibrosis in chronic HCV infection [15].

Cellular immune responses influence a response to treatment and contribute to liver pathology. Viral clearance during treatment was associated with low type 1 (T1)/type 2 (T2) ratios before starting therapy [19,29]. Pre-treatment serum levels of a T-cell activation marker, CD30, may be higher in patients who achieved a sustained virological response [14]. Furthermore IFN$\gamma$ responses to HCV antigens by peripheral blood mononuclear cells are lowest in patients with advanced fibrosis [2,34], suggesting high T1 responses are required to promote clearance of the virus and/or inhibit hepatic fibrosis. The current view is that T1 responses assist viral clearance and lead to inflammation, whilst T2 responses (notably IL-13) promote fibrosis.

Here we assess cytokines involved in the regulation of fibrosis [IL-13R$\alpha$2 and transforming growth factor (TGF)-$\beta$1] or in the interferon-$\gamma$ (IFN$\gamma$) pathway (IL-18 and IP-10), and a marker of T-cell activation (soluble CD30). We addressed whether a combination of Hepascore and these markers could predict response to pegIFN$\alpha$/RBV combination therapy and/or assess fibrosis.

## 2. Materials and methods

### 2.1. Patients

Serum and plasma samples were collected and liver biopsies were performed on 95 consecutive patients assessed for treatment of chronic HCV infection at Royal Perth Hospital (Western Australia) between 2002 and 2005. Samples tested were collected a few days before treatment. The median time between liver biopsies and collection of samples was 2 months (interquartile range 1–7 months). Median length of liver biopsy specimens was 15 mm (interquartile range 12–18 mm). Fibrosis was evaluated according to the Scheuer scoring systems [27] and staged as: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, portal fibrosis with many septa; F4, cirrhosis. Patients were grouped as absent or minimal fibrosis (F0,F1) or 'significant fibrosis' (F2, F3 or F4).

All patients received pegylated IFN$\alpha$ (180 $\mu$g subcutaneous injection per week, Pegasys, Roche, Dee Why, NSW, Australia or 1.5 $\mu$g/kg subcutaneous injection per week. Pegatron, Schering-Plough, North Ryde, NSW, Australia) and 1200–1800 mg ribavirin combination therapy. HCV genotypes 1 or 4 were treated for 48 weeks and genotypes 2 or 3 were treated for 24 weeks. Patients were classified as sustained virological responders (SVR) if their serum HCV RNA was undetectable ($<$ 10 IU/ml; Cobas Amplicor HCV Test, Roche Diagnostics, USA) 24 weeks after end-of-treatment. The study was approved by the Royal Perth Hospital Research Ethics committee and all patients gave written informed consent.

### 2.2. Biochemical markers

Serum ALT, bilirubin and GGT levels were measured on an automated analyzer (Hitachi 917; Roche Diagnostics, USA). Hyaluronic acid (HA) was measured by ELISA (Corgenix, UK).$\alpha_2$-macroglobulin levels were obtained by nephelometry (Immage; Beckman Coulter, USA). Hepascore was calculated as described previously [1] using the following algorithm Hepascore $= y/(1 + y)$ where $y =$ exp [-4.1858 $-$ (0.0249 x age) $+$ (0.7464 x sex) $+$ (1.0039 x $\alpha$2-macroglobulin) $+$ (0.0302 x hyaluronic acid) $+$ (0.0691 x bilirubin) – (0.0012 x GGT)].

### 2.3. Immunological markers

Commercial antibody pairs were used to quantitate serum sCD30 (Bender MedSystems, Austria), serum IL-13R$\alpha$2, plasma TGF$\beta$1 (R&D Systems; MN, USA) and plasma IL-18 (MBL, Japan) by ELISA. Active and total TGF$\beta$1 were measured in plasma samples after centrifugation (11,000rpm, 20 mins) to deplete platelets. Total TGF$\beta$1 was measured by activating latent TGF$\beta$1 by acid treatment. IP-10 (CXCL10) was measured by Cytokine Bead Array (BD Biosciences; NJ, USA). Briefly, plasma was mixed with capture beads and mouse phycoerythrin detection reagent, incubated, washed and resuspended in wash buffer before acquisition. The limits of detection and coefficient of variance for each assay were sCD30 (1.6 U/mL, 15%), IL-13R$\alpha$2 (7.0 pg/mL, 7.3%), TGF$\beta$1 (0.43 pg/mL, 10%), IL-18 (16 pg/mL, 7.4%) and IP-10 (2.3 pg/mL, 4.0%).

### 2.4. Statistics

Results are given as number (percentage) or median (range). Univariate comparisons used Mann-Whitney or Fisher's Exact Tests. Associations between serological markers and fibrosis or treatment outcome were assessed using multiple logistic regression modelling including all factors with $p < 0.3$ on univariate anal-

yses. Diagnostic accuracy was assessed using the Receiver Operating Characteristic (ROC) Area Under the Curve (AUC) analysis. Combinations of markers with the highest AUC were identified. Differences between AUC values were evaluated using $\chi^2$ tests.

The logistic regression model consisted of:

$$y = \text{Exp}[1.1163 + (0.0052 \text{ x Age}) - (0.1943 \text{ x Gender}) - (1.5908 \text{ x HCV genotype}) + (0.063 \text{ x Total TGF}\beta 1) - (0.0017 \text{ x IP-10})]$$

with age provided in years, male sex $= 1$, female sex $= 0$, HCV genotype [1a,1b,4] $= 1$ and HCV genotype [2a,2a,3a,3b] $= 0$.

TIPscore was calculated using the following equation: y/y + 1

Sensitivity and specificity were calculated for treatment outcome and fibrosis. Multivariate analyses used Stata Version 10 (Stata Corporation; TX, USA). In all analyses, $p$-values $\leqslant 0.05$ are considered statistically significant and $0.05 < p < 0.10$ is noted as suggesting a trend.

## 3. Results

### 3.1. Immunological factors did not associate individually with treatment outcome or fibrosis

Of the 95 patients enrolled, virological response 24 weeks after end-of-treatment was known for 88 patients who completed pegIFN$\alpha$/RBV therapy. A sustained virological response (SVR) was achieved by 52 (59%) of the 88 patients. Additionally, HCV viral load data at week 12 was available for 58 of the 88 patients (63%). Early virologic response (EVR) was observed for 33 patients (57%) who all achieved a SVR. There were 25 (43%) non-EVR and 4 of these patients achieved a SVR. Levels of immunological markers were statistically similar in SVR and non-SVR prior to treatment (Table 1), but median levels of IP-10 were slightly lower and total TGF$\beta$1 levels were slightly higher in SVR. This was explored in the multivariable model.

Levels of the immunological markers of fibrosis were also compared with stages of fibrosis (Table 2). In a univariate analysis, there was a trend towards higher IL-18 levels in patients with significant fibrosis (F2–F4) than patients with F0/F1 scores ($p = 0.08$).

### 3.2. Biochemical and virological factors associated with treatment outcome and fibrosis

Infection with HCV genotype (G) 1 or 4 was associated with poor treatment outcome ($p = 0.01$), and weak associations were noted with GGT and bilirubin levels ($p = 0.07$ and $p = 0.05$, respectively) (Table 1). Response to treatment was not associated with sex, age or Hepascore. Classification of patients into those with significant (F2–F4; Table 1) or advanced (F3,F4; data not shown) fibrosis did not predict treatment outcome.

Patients with significant fibrosis (F2–F4) were older and had significantly higher GGT, HA and $\alpha_2$-macroglobulin levels than patients with F0,F1 fibrosis scores (Table 2). Higher Hepascore were observed in patients with significant fibrosis, a finding consistent with our previous study [1].

### 3.3. An algorithm including IP-10 and TGF$\beta$ (*TIPscore*) best predicts treatment outcome

Using ROC analysis, Hepascore was a poor predictor of treatment outcome (Table 3). Addition of all immunological markers improved prediction based on Hepascore alone ($p = 0.04$) with a sensitivity of 79%, but the specificity remained poor (36%) based on a cut-off score of 0.5. As levels of TGF$\beta$1 (total) and IP-10 weakly associated with treatment outcome in a univariate analysis ($p \leqslant 0.2$), they were tested alone and with other factors. The optimum model (termed "TIPscore", Fig. 1a) was based on levels of TGF$\beta$1 (total) and IP-10, corrected for age, sex and HCV genotype.

TIPscore was significantly better than HCV genotype alone for the prediction of response to therapy [(Genotype AUC $= 0.67$) *vs* (TIPscore AUC $= 0.76$), $p = 0.03$] and marginally better than HCV genotype adjusted for age and sex ($p = 0.07$). It predicted outcome with a sensitivity of 81% and a specificity of 58% ($p = 0.003$ relative to Hepascore). The highest probability of correct classification of all patients in our cohort (69%) was achieved with a TIPscore of 0.557 (sensitivity 78%, specificity 56%). TIPscores were significantly higher in SVR than non-SVR (Fig. 1b; Mann Whitney test, $p = 0.0002$).

### 3.4. TIPscore is inferior to Hepascore for assessment of fibrosis

Hepascore effectively predicted significant fibrosis with a sensitivity of 78% and a specificity of 79% using a cut-off score of 0.5 (Table 3). A combination of all im-

Table 1
Association of immunological and biochemical markers with treatment outcome in chronically HCV-infected patients receiving IFN$\alpha$ and ribavirin therapy

| | SVR ($n = 52$) | Non-SVR ($n = 36$) | $p$-value[a] |
|---|---|---|---|
| Male (%) | 31 (60%) | 23 (64%) | 0.82 |
| Age (years) | 46 (23–76) | 48 (23–68) | 0.26 |
| Race | | | |
| Caucasian | 45 | 27 | |
| Asian | 7 | 9 | 0.26 |
| HCV genotype[b] | | | |
| G1,G4 | 21 | 24 | |
| G2,G3 | 27 | 9 | 0.01 |
| Scheuer stage[c] (%) | | | |
| F0,1 | 38 | 25 | |
| F2-4 | 14 | 11 | 0.64 |
| *Immunological markers* | | | |
| CD30 (U/mL) | 61 (22–224) | 61 (23–204) | 0.93 |
| IL-13R$\alpha$2 (pg/mL) | 0 (0–112242) | 0 (0–9792) | 0.49 |
| Total TGF$\beta$1 (ng/mL) | 11 (2.3–61) | 7.4 (2.9–26) | 0.16 |
| Active TGF$\beta$1 (pg/mL) | 84 (0–1181) | 71 (16–736) | 0.87 |
| IL-18 (pg/mL) | 753 (224–4969) | 777 (330–3626) | 0.94 |
| IP-10 (pg/mL) | 254 (99–975) | 317 (52–1411) | 0.20 |
| *Biochemical markers* | | | |
| ALT (IU/mL) | 96 (21–449) | 90 (25–723) | 0.46 |
| GGT (U/L) | 46 (10–677) | 72 (13–457) | 0.07 |
| Bilirubin ($\mu$mol/L) | 9 (3–53) | 12.5 (5–22) | 0.05 |
| Hyaluronic acid ($\mu$g/L) | 42 (3.9–800) | 32 (1.0–386) | 0.27 |
| $\alpha_2-$macroglobulin (g/L) | 2.9 (1.2–5.5) | 3.2 (1.4–6.0) | 0.91 |
| Hepascore | 0.47 (0.09–1.00) | 0.56 (0.08–1.00) | 0.77 |

[a]Fisher's Test or Mann-Whitney Test.
[b]Data not available for 4 responders and 3 non-responders.
[c]Data not available for 2 non-responders.

Table 2
Association of age, sex, biochemical and immunological markers with significant fibrosis in chronically HCV-infected patients

| | Fibrosis stage F0/F1 ($n = 36$) | Fibrosis stage F2–F4 ($n = 59$) | $p$-value[a] |
|---|---|---|---|
| Male (%) | 20 (56%) | 40 (68%) | 0.28 |
| Age (years) | 43 (23–57) | 47 (23–76) | 0.02 |
| *Immunological markers* | | | |
| CD30 (U/mL) | 60 (23–196) | 61 (22–224) | 0.80 |
| IL-13R$\alpha$2 (pg/mL) | 0 (0–3534) | 0 (0–112242) | 0.68 |
| Total TGF$\beta$1 (ng/mL) | 9.1 (2.9–61) | 10 (2.3–54) | 0.92 |
| Active TGF$\beta$1 (pg/mL) | 70 (6–1180) | 97 (0–1181) | 0.50 |
| IL-18 (pg/mL) | 708 (224–3626) | 802 (440–4969) | 0.08 |
| IP-10 (pg/mL) | 260 (74–1411) | 283 (52–975) | 0.33 |
| *Biochemical markers* | | | |
| ALT (IU/mL) | 68 (14–723) | 98 (25–401) | 0.12 |
| GGT (U/L) | 39 (5–384) | 66 (16–677) | 0.004 |
| Bilirubin ($\mu$mol/L) | 9.5 (3–29) | 11 (5–53) | 0.40 |
| Hyaluronic acid ($\mu$g/L) | 30 (3–164) | 48 (1–800) | 0.0001 |
| $\alpha_2-$macroglobulin (g/L) | 2.5 (1.2–4.8) | 3.7 (1.2–6.0) | 0.0001 |
| Hepascore | 0.32 (0.10–0.97) | 0.73 (0.08–1.00) | $< 0.0001$ |

[a]Fisher's Exact Test (categorical variables) or Mann-Whitney Test (continuous variables).

munological markers yielded better sensitivity (85%) but the specificity was poor (28%). TIPscore also had a low AUC as a marker of significant fibrosis. This was inferior to Hepascore ($p = 0.03$).

## 4. Discussion

Liver damage and fibrosis are mediated by cellular immune responses and advanced fibrosis is associat-

Table 3
ROC analysis of the capacity of Hepascore and immunological markers to predict fibrosis and treatment outcome

| | AUC | Sensitivity (%)[a] | Specificity (%)[a] | Correctly classified (%)[a] | *p*-value |
|---|---|---|---|---|---|
| *Virological response to treatment* | | | | | |
| Hepascore alone (Hep) | 0.52 | 100 | 0 | 59 | |
| All immunological markers (All) | 0.69 | 77 | 36 | 60 | 0.04 |
| Hep + All | 0.66 | 79 | 36 | 61 | 0.04 |
| HCV genotype (G) + age & sex | 0.65 | 61 | 67 | 64 | 0.10 |
| TGF$\beta$1(tot), IP-10 | 0.66 | 83 | 31 | 61 | 0.09 |
| Hep + TGF$\beta$1(got), IP-10 | 0.67 | 85 | 31 | 62 | 0.09 |
| TGF$\beta$1(tot), IP-10 + age & sex | 0.68 | 85 | 31 | 62 | 0.04 |
| TGF$\beta$1(tot), IP-10 + G, age & sex | 0.76 | 81 | 58 | 72 | 0.003 |
| *Significant Fibrosis* (*Scheuer F2–F4*) | | | | | |
| Hep | 0.80 | 77 | 79 | 78 | |
| All | 0.65 | 85 | 28 | 61 | 0.13 |
| Hep + All | 0.86 | 77 | 72 | 75 | 0.18 |
| HCV genotype (G) + age & sex | 0.71 | 85 | 38 | 65 | 0.04 |
| TGF$\beta$1(tot), IP-10 | 0.48 | 100 | 3 | 59 | 0.002 |
| Hep + TGF$\beta$1(tot), IP-10 | 0.80 | 75 | 76 | 75 | 0.92 |
| TGF$\beta$1(tot), IP-10 + age & sex | 0.69 | 82 | 38 | 64 | 0.17 |
| TGF$\beta$1(tot), IP-10 + G, age & sex | 0.70 | 90 | 31 | 65 | 0.03 |

[a]Based on a cut-off score of 0.5 for each algorithm.

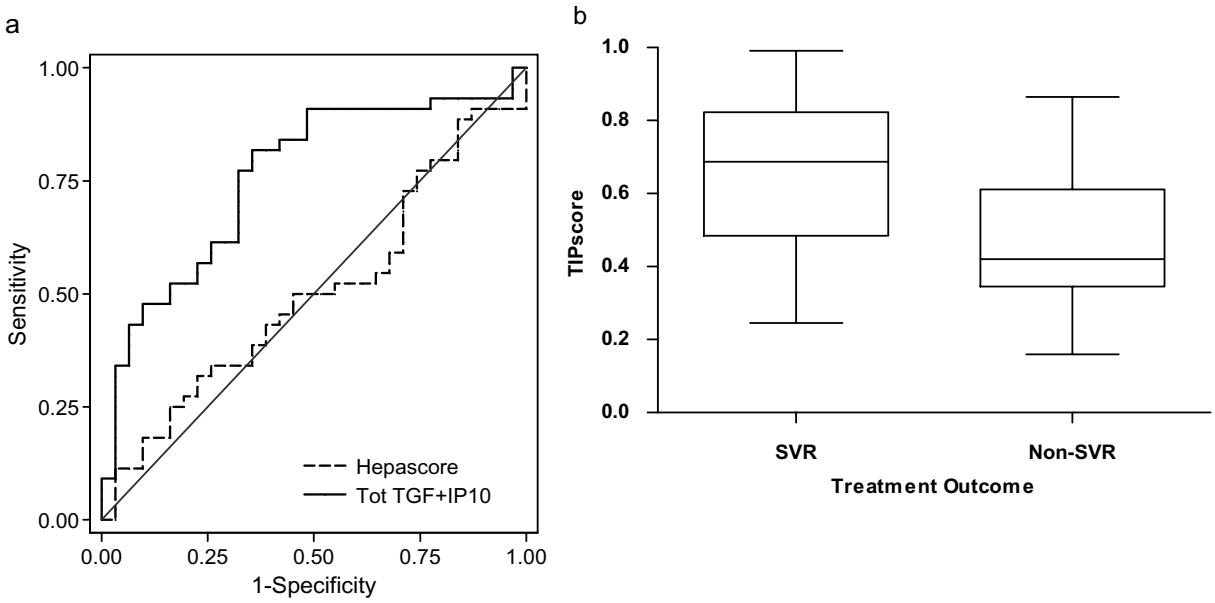


Fig. 1. Evaluation of the optimal model for predictiing treatment outcome ("TIPscore"). a. ROC curves for Hepascore and a TIPscore calculated as y/y + 1, where $y$ = Exp[1.1163 + (0.0052 x Age) – (0.1943 x Gender) – (1.5908 x HCV Genotype) + (0.063 x Total TGF$\beta$1) – (0.0017 x IP-10)] Gender: 1 = Male, 0 = Female; Genotype: 1 = [1a,1b,4], 0 = [2a 2a,3a,3b]. b. Boxplot of TIPscore according to treatment outcome.

ed with poor treatment outcome. Hence we assessed serological markers that reflect a cytokine environment likely to favour T-cell mediated fibrogenesis or inflammation as predictors of response to treatment and markers of fibrosis. The approach had potential to include fibrosis in prediction of a response to treatment without a liver biopsy. Previously positive predictive values of 95% were obtained with an algorithm based on sex, age, prior treatment status, pretreatment serum ALT and HCV RNA, and METAVIR scores [17].

Samples, longitudinal clinical data and Scheuer scores were available from 88 patients (52 SVR and 36 non-SVR). With no reliable estimates of individual variation to feed into a power calculation, we note that

this is a larger cohort than previous studies correlating plasma markers with biopsy data and treatment outcome [32,33]. However sample size is acknowledged as a limitation to our study

We validated Hepascore for the assessment of fibrosis, but found poor specificity in the prediction of treatment outcome. The most potent single predictor of response to therapy was viral genotype. However an algorithm based on levels of total TGF$\beta$1 and IP-10 adjusted for viral genotype, age and sex was more effective than genotype alone and slightly better than genotype adjusted for age and sex. Whilst there is no precedent for consideration of TGF$\beta$1 and IP-10 together, both are implicated in the pathogenesis of chronic HCV disease. IP-10 (CXCL10) is a chemokine induced by IFN$\gamma$ [38] and involved in leukocyte recruitment via CXCR3. IP-10 can be produced by hepatocytes and up-regulation of expression in the liver during chronic HCV infection correlates with the accumulation of T lymphocytes expressing CXCR3 [9]. Elevated serum and intrahepatic levels of IP-10 are reported in patients with HCV genotype 1 and are associated with liver damage and failure to respond to HCV therapy [13, 26]. Pre-treatment IP-10 levels also predict treatment outcome in patients co-infected with HIV [25,37]. A model based on baseline viral load, gender, body mass index and IP-10 levels yielded predictive values of 79–86% in a cohort of 173 European HCV patients with HCV genotype 1 [13]. The authors emphasized the need to incorporate viral genotype in any predictive model. Whilst they noted that age affected response to treatment, it was not in their model. A study focusing on patient age showed the effect of genotype was restricted to patients aged 35–55 years [5].

TGF$\beta$1 is a central mediator of fibrosis which promotes synthesis of collagen type I, stellate cell migration and hepatocyte apoptosis [4]. TGF$\beta$1 levels in plasma decline after treatment with IFN$\alpha$ therapy with regression of liver fibrosis [31]. Baseline levels of TGF$\beta$1 have not been associated with outcome of treatment, but alleles of polymorphisms in codons 10 and 25 associated with treatment outcome in HIV/HCV co-infected patients [21]. These alleles had been linked with elevated production of TGF$\beta$1 *in vitro*. Serum levels of TGF$\beta$1 were higher in HCV patients than controls and declined by 25% on therapy, irrespective of treatment outcome [12]. These values are higher than obtained in plasma here, and show surprisingly little individual variation.

We considered a mechanism by which low levels of IP-10 and high levels of TGF$\beta$1 may aid control of HCV replication by IFN$\alpha$ and ribavirin. TGF$\beta$1 can suppress HCV replication in an *in vitro* replicon system, by signaling through SMAD transcription factors rather than via MAP kinases [20]. High levels of IP-10 were seen in HIV/HCV co-infected patients, particularly in association with a poor response to therapy [37]. Hence an association of low IP-10 and high TGF$\beta$x with a favourable outcome is plausible.

The T2 cytokine, IL-13 can increase collagen synthesis in cultured human hepatic stellate cells via interaction with its high affinity receptor (IL-13R$\alpha$2) [30]. IL-13R$\alpha$2 can exist as a soluble form *in vivo*, acting as a soluble decoy receptor for IL-13 [35]. Blocking IL-13 activity with IL-13R$\alpha$2 can reduce liver fibrosis [18]. In chronic HCV infection, pretreatment IL-13 levels were associated with an early virologic response and levels declined during pegIFN$\alpha$ and ribavirin treatment [7]. Ours is the first study to measure IL-13R$\alpha$2 in serum from patients with chronic HCV infection. However, most patients had undetectable IL-13R$\alpha$2 and serum levels were not associated with treatment outcome or fibrosis. Intracellular and membrane IL-13R$\alpha$2 expression may better reflect IL-13 responses.

CD30 is a member of the tumour necrosis factor receptor family expressed by activated CD4 and CD8 T-cells. HCV patients with a virological response to standard IFN$\alpha$ and ribavirin therapy exhibited high serum levels of sCD30 before treatment and a significant decline on therapy [14,36]. Here sCD30 levels were not associated with treatment outcome or severity of fibrosis, perhaps because some patients who did not respond to standard IFN$\alpha$ may respond to pegIFN$\alpha$.

IL-18 is a pro-inflammatory cytokine primarily produced by activated macrophages. It acts synergistically with IL-12 to promote IFN$\gamma$ production by T-cells. Since IFN$\gamma$ is not measurable in plasma, IL-18 is interesting as a marker of this pathway. Levels are upregulated in HCV patients compared to healthy controls and are associated with inflammation [22] rather than fibrosis [28]. Here, baseline IL-18 levels did not predict treatment outcome but high levels were observed in patients with advanced fibrosis (F4, cirrhosis). High IL-18 levels were similarly associated with disease progression in patients with alcoholic-induced cirrhosis [8] or cirrhosis due to different etiologies [16].

In conclusion, we confirm the utility of Hepascore for the assessment of fibrosis and describe an algorithm for the prediction of outcome to pegIFN$\alpha$ and ribavirin treatment based on HCV genotype, the patient's age and sex and pretreatment levels of IP-10 and TGF$\beta$1

in plasma. Importantly, the study associates low IP-10 and high TGF$\beta$x levels with a virological response to therapy. This profile favours fibrogenesis over inflammation.

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