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Twelve Potential Fibrosis Markers to Differentiate Mild Liver Fibrosis from Cirrhosis in Patients Infected with Chronic Hepatitis C Genotype 1

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Abstract

Information of the stage of liver fibrosis is important for managing patients with chronic hepatitis C (CHC). The aim of this study was to evaluate twelve plasma markers for differentiating no/mild liver fibrosis from cirrhosis among patients with CHC, genotype 1.

Transient elastography was used to assess the stage of fibrosis for the patients included. Forty patients were included (21 cirrhotic). Plasma levels of TNF- α (tumor necrosis factor α), IL-8 (interleukin 8), IP-10 (interferon-g inducible protein 10), MCP-1 (monocyte chemotactic protein-1), suPAR (soluble urokinase-type plasminogen activator), MIG (monokine induced by γ -interferon), HGF (human hepatocyte growth factor), insulin, IL-6 (interleukin 6), IL-2 β (interleukin 2- β), leptin and NGF (nerve growth factor) were analyzed.

Concentrations of TNF- α (median 15.0 vs. 25.1 pg/ml, Area Under Receiver Operating Characteristic Curve (AUC) 0.91), IL-8 (48.7 vs. 103.3 pg/ml, AUC 0.85), IP-10 (176 vs. 566 pg/ml, AUC 0.83), MCP-1 (449 vs. 735 pg/ml, AUC 0.78), suPAR (3.5 vs. 5.2 ng/ml, AUC 0.78), MIG (100 vs. 152 pg/ml, AUC 0.75) and HGF (3.69 vs. 5.58 ng/ml, AUC 0.71) were significantly higher in patients with cirrhosis. In conclusion, several of the investigated markers showed promise for differentiating cirrhosis from no/mild fibrosis among patients with CHC genotype 1.

Introduction

Worldwide, approximately 170 million people are infected with chronic hepatitis C (CHC) [1]. Mortality associated with CHC results mainly from development of fibrosis and subsequent cirrhosis [2]. Accurate staging of liver fibrosis in patients with CHC is important for prognostic information and guides the physician in treatment decisions. Traditionally monitoring of fibrosis has been based on histological staging of liver biopsies, a procedure that is both unpleasant for the patient and may have severe side effects [3;4]. Transient elastography (TE) has within the last few years been widely accepted for its excellent diagnostic accuracy for fibrosis and cirrhosis for patients infected with CHC [5-8], but this method requires trained staff and it may be difficult to obtain a valid examination in obese patients.

CHC results in a process of inflammation and progressive fibrosis [2]. Pro-inflammatory cytokines and chemokines are involved in this process and plasma levels of these may be associated with different stages of hepatitis C associated liver fibrosis [9;10]. Although current knowledge about these inflammatory cytokines and chemokines is limited, studies have demonstrated that plasma levels of some chemokines correlate with liver fibrosis [11-13]. There is a need for non-invasive, simple, inexpensive and accurate tools to determine and monitor liver fibrosis in CHC patients. Chemokines, cytokines and other plasma proteins are easily obtainable from a blood sample and are an attractive possibility.

In this study, we have investigated the potential of using plasma levels of TNF- α (tumor necrosis factor- α), IL-8 (interleukin 8, CXCL8), IP-10 (interferon- γ inducible protein 10, CXCL10), MCP-1 (monocyte chemotactic protein-1, CCL2), suPAR (soluble urokinase-type plasminogen activator), MIG (monokine induced by γ -interferon, CXCL9), HGF (human hepatocyte growth factor), insulin, IL-6 (interleukin 6), IL-2 β (interleukin 2 β), leptin and NGF (nerve growth factor) for differentiating no/mild liver fibrosis from cirrhosis in patients with CHC genotype 1.

Methods

Patient and samples collection

Patients with CHC genotype 1 (Hepatitis C-RNA PCR detectable for more than six months) were

included in the study if they had no/mild fibrosis or cirrhosis determined by a successful examination with TE. Including only patients with CHC and with no/mild fibrosis or cirrhosis was done to have a better probability to detect a correlation between potential markers and the stage of fibrosis in this initial study. Patients were recruited between January 2009 and March 2010 from two Danish clinics specialized in hepatitis. At the time of examination with TE, blood was drawn for hepatitis C RNA detection, platelet counts, analysis of alanine-aminotransferase (ALT) and a plasma sample was frozen for later analysis of potential fibrosis markers. To be able to detect a statistical significant difference between the two groups, we planned to include 40 participants and the study was closed for inclusion when 40 patients were reached.

The study has been approved by The Danish National Committee on Biomedical Research Ethics (H-D-2007-0087) in accordance with the Helsinki Declaration. All patients had given written and oral consent to participate in the study.

Inclusion criteria

Patients, older than 18 years of age, with CHC genotype 1 and with no/mild liver fibrosis (<7.7 kPa) or cirrhosis (≥ 13.0 kPa), were offered participation in the study.

Exclusion criteria

Patients with hepatocellular carcinoma, previous liver transplantation, or liver metastases were not included in the study. Patients, co-infected with hepatitis C virus genotypes different from 1, with hepatitis B virus or with human immunodeficiency virus (HIV), were not offered inclusion in the study. Furthermore, patients were excluded if they had been administered interferon or ribavirin for up to one year before examination. Patients with a self-reported current daily large alcohol consumption (>36 grams women, >60 grams males) were not included.

Transient elastography

For liver stiffness measurements we used FibroScan® (Echosens, Paris, France) with a medium probe and software version 1.30. Liver stiffness measurement was considered successful if ten valid measurements were obtained (valid measurements $>60\%$ and interquartile range $<25\%$). Patients were

diagnosed as having no/mild fibrosis when liver stiffness was below 7.7 kPa, and as having cirrhosis when the values were equal to or above 13.0 kPa. These cut-off values were selected prior to the study and are based on a large meta-analysis including 50 studies of correlation between fibrosis determined by liver biopsy and transient elastography [7]. For diagnosing cirrhosis (F4 Metavir classification) with a cut-off value of 13.0 kPa, a mean AUC (Area Under Receiver Operating Characteristic Curve) of 0.94 has been shown and for diagnosing significant fibrosis (F2) with liver stiffness above 7.7 kPa, the mean AUC was 0.84 [7]. Liver stiffness measurements were performed by two physicians certified in the use of TE and with experience of more than 300 examinations.

Test methods

Plasma from one 9 ml EDTA coated tube was separated by centrifugation and stored at -80°C until fibrosis marker measurements were performed. Blood samples were taken from patients at the same day of liver stiffness measurement.

All fibrosis markers were measured blinded and in duplicates. Plasma levels of IL-2 β , IL-6, IL-8, insulin, HGF, leptin, MCP-1, NGF, TNF- α were determined per protocol using Luminex xMAP technology (Luminex corp., Austin, TX, USA) and Miliplex Map Human Serum Adipokine (Panel B) Kit in 1:1 diluted samples (Millipore Corp., St. Charles, MO, USA). Plasma levels of suPAR, IP-10 and MIG were determined with enzyme-linked immunosorbent assays (ELISA) in accordance with the manufacturer's instructions (suPARnostic, ViroGates, Denmark; Quantikine, R&D Systems, MN, USA). IP-10 levels were also determined with an in-house IP-10 ELISA (provided by M. Ruhwald). ELISA of suPAR was done after diluting plasma 1:10, IP-10 after diluting plasma samples 1:4 and MIG after dilution 1:1. Measurements were fitted with linear regression analysis. All fibrosis marker concentrations are presented after correcting for dilution.

Statistics

Concentrations of fibrosis markers were compared using non-parametric tests. The diagnostic performance of the markers was determined using ROC curve analysis and compared in accordance with the method suggested by Hanley and McNeil [14]. Data were analyzed using the software "R Statistics", version 2.9.0 (R Development Core Team, Vienna, Austria) and SPSS version 17.0 (SPSS

Inc., Chicago, IL, USA). P-values<0.05 were considered significant.

Results

Among the 40 patients included, 21 had cirrhosis and 19 had no/mild fibrosis. The median age was 57 years for the cirrhotic patients versus 46 years for the patients with no/mild fibrosis. Furthermore, the cirrhotic patients had a median body mass index (BMI) of 26.5 versus 22.6 for the non-cirrhotic patients (Table 1).

Of the 40 patients included, 10 had within 5 years of inclusion undergone a percutaneous liver biopsy of minimum 10 mm in length containing at least five portal tracts. Local pathologists had independently of this study evaluated the biopsy. Among these patients, 9 (90%) had a fibrosis stage determined by liver biopsy in agreement with the fibrosis stage determined by TE.

Concentrations of the twelve potential fibrosis markers were compared between patients with no/mild fibrosis and cirrhosis. TNF- α , IL-8, IP-10, MCP-1, suPAR, MIG and HGF were significantly higher in cirrhotic patients compared to patients with no/mild fibrosis. There were no differences in the IP-10 levels measured with the commercial and the in-house ELISA, therefore only the levels determined with the in-house assay is presented. Concentration levels of the twelve markers for patients with no/mild fibrosis and cirrhosis, respectively, are shown in Figure 1. The ROC curves for TNF- α , IL-8, IP-10, MCP-1, and suPAR are illustrated in Figure 2. The fibrosis markers that differentiated best between the two groups were TNF- α (median 15.0 vs. 25.1 pg/ml, AUC 0.91), IL-8 (48.7 vs. 103.3 pg/ml, AUC 0.85), and IP-10 (176 vs. 566 pg/ml, AUC 0.83) (Table 2). There were no significant differences in AUCs between any of the fibrosis markers that showed significantly association with fibrosis stage.

Discussion

The present case control study shows that plasma levels of TNF- α , IL-8, IP-10, MCP-1, suPAR, MIG, HGF are significantly higher for cirrhosis than with no/mild fibrosis among patients with chronic hepatitis C, genotype 1. Especially TNF- α , IL-8 and IP-10 show potential as fibrosis markers as they were consistently expressed in high levels in patients with cirrhosis and not in patients with no/mild

fibrosis.

An ideal fibrosis marker should not only have a high sensitivity and specificity, it must also be easy assessable, inexpensive, accurate and reproducible. Fibrosis markers from a blood sample are easily obtained and therefore an appealing possibility to search for markers to fulfill the remaining criteria. Some of the potential fibrosis markers in this study have earlier been investigated. Moura et al. have recently demonstrated that plasma concentration of MIG was significantly higher among patients with CHC and moderate fibrosis than with absent/mild fibrosis but unlike the presented study, they were not able to show any significant difference between plasma levels of IP-10 and MCP-1 for the two groups. This could be due to the fact that Moura et al. compared absent/mild fibrosis with moderate fibrosis ($\geq F2$) instead of cirrhosis combined with a relatively limited number of patients included [13]. In agreement with our findings, MCP-1 and IP-10 have in other studies shown to be associated with the stage of liver fibrosis among patients with CHC [12;15;16]. TNF- α seems to be associated with liver inflammation but another study have also found that TNF- α also is associated with the stage of fibrosis among patients with chronic hepatitis C in correlation with our results [17-19]. Interestingly, plasma levels of IL-8 seems to be associated with tumor size and stage of hepatocellular carcinoma which indicates that besides having potential as a fibrosis markers, IL-8 may also be involved in progression of hepatocellular carcinoma [20;21]. Association between plasma levels of HGF for patients with CHC and no/mild fibrosis or cirrhosis have been demonstrated in an earlier study and the findings are in agreement with ours [22]. The association between suPAR and cirrhosis among patients with CHC has to our knowledge not been demonstrated earlier. Elevated plasma suPAR level has been associated with faster disease progression and increased risk of mortality in both diseased patients and in the general population [23].

A limitation of the study was that we only demonstrated a binary distinction between no/mild fibrosis and cirrhosis. The possibility of using these markers to differentiate stages of fibrosis could provide additional prognostic information. Furthermore, combining information from several markers may improve diagnostic accuracy in detecting liver fibrosis. This study was underpowered to investigate a significant effect of combining the investigated fibrosis markers. So far, no combined plasma fibrosis markers have gained universal acceptance even though several have been proposed e.g. Fibro Test,

Forns' Fibrosis Index and APRI [24-26]. In this study, we find individually fibrosis markers fully comparable with these indexes e.g. APRI (with a cut-off of >1.0, AUC 0.89 in training set/0.94 in validation set for diagnosing cirrhosis) and Forns' Fibrosis Index (with a cut-off of >4.2, AUC of 0.86 in estimation group/0.81 validation group for diagnosing significant fibrosis) [24;26]. A combined fibrosis marker based on some of the markers investigated in this study may be superior to these fibrosis indexes but further studies are needed.

Several studies have demonstrated a strong correlation between liver stiffness and the stage of fibrosis determined by liver biopsy [5-8]. Two studies have found that TE is unsuitable for detecting fibrosis in patients with acute hepatitis involving severe inflammation, since this increases liver stiffness [27;28]. Patients with CHC could potentially have a flare-up in liver inflammation, when included in this study, resulting in a higher liver stiffness and causing overestimation of the fibrosis stage. In addition, liver inflammation has been shown to result in higher levels in some chemokines [10;13]. Severe inflammation could therefore give both higher liver stiffness values and plasma levels and be misinterpreted as pronounced liver fibrosis. However, ten of the 40 patients included had also undergone a liver biopsy showing good correlation between fibrosis stages determined by liver biopsy and by TE (90%).

The present study was cross-sectional and thus unable to provide information on the potential of the fibrosis marker measurements for monitoring fibrosis changes and prediction of treatment outcome. Recently, studies have demonstrated that one of the investigated fibrosis markers (IP-10) provides prognostic information on treatment outcome in patients mono-infected with CHC as well as HIV/CHC co-infected [29-32]. In a study with 173 patients with CHC genotype 1 included, pre-treatment IP-10 levels predicted rapid and sustained virological response [32].

In summary, in a patient sample of 40 individuals, we find that plasma concentrations of TNF- α , IL-8, IP-10, MCP-1, suPAR, MIG and HGF are significantly higher for cirrhosis than no/mild liver fibrosis in patients infected with CHC, genotype 1. Insulin, IL-6, IL-2 β , leptin, NGF do not seem to be significantly correlated to fibrosis in our patient sample. Especially TNF- α , IL-8 and IP-10 show potential as suitable fibrosis markers as they were consistently expressed in high levels in patients with

advanced fibrosis and not in patients without fibrosis. This study was an initiative study and further prospective studies are needed among patients with CHC to validate the clinical applicability of these fibrosis markers alone or in combination.

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Potential Conflicts of Interest

Belinda Klemmensen Moessner has received an unrestricted research grant from Roche A/S. Peer Brehm Christensen is a member of the advisory board for Roche A/S and has received research grants from Roche and Scheering-Plough A/S. Roche A/S provided the Fibroscan device used in this study. Jesper Eugen-Olsen is a founder and board member of ViroGates A/S.

Table 1 Characteristics of 40 patients with chronic hepatitis C, genotype 1, and either no/mild Fibrosis or Cirrhosis

	No/mild Fibrosis	Cirrhosis	P-value ^d
Patients ^a	19 (47.5%)	21 (52.5%)	
Age (years) ^b	46 (38; 54)	57 (51; 61)	0.001
Gender (males) ^a	8 (42%)	15 (71%)	0.117
BMI (kg/m ²) ^b	22.6 (20.4; 26.1)	26.5 (23.9; 29.9)	0.019
Liver Stiffness (kPa) ^b	4.9 (4.7; 6.1)	23.2 (16.9; 32.3)	
ALT (IU/l) ^{b,c}	38 (27; 59)	94 (52; 144)	>0.000
Platelets counts (10 ⁹ /l) ^b	242 (188; 260)	144 (107; 205)	>0.000

^a Number (%)

^b Median with 25 and 75 percentiles in bracket

^c Alanine aminotranferase

^d Mann Whitney test for equality

Table 2 Median concentration of TNF- α (tumor necrosis factor- α), IL-8 (interleukin 8), IP-10 (interferon- γ inducible protein 10), MCP-1 (monocyte chemotactic protein-1), suPAR (soluble urokinase-type plasminogen activator), MIG (monokine induced by γ -interferon), HGF (human hepatocyte growth factor), insulin, IL-6 (interleukin 6), IL-2 β (interleukin 2- β), leptin, NGF (nerve growth factor) for 40 patients with chronic hepatitis C, genotype 1, and either no/mild fibrosis or cirrhosis. 25 and 75 percentiles are shown in brackets.

Serum Markers	Liver Fibrosis		P-value ^a	AUC ^b
	None/Mild Fibrosis	Cirrhosis		
TNF- α (pg/ml)	15.0 (13.2; 17.6)	25.1 (20.3; 32.6)	>0.000	0.91
IL-8 (pg/ml)	48.7 (31.8; 56.8)	103.3 (75.9; 148.3)	>0.000	0.85
IP-10 (pg/ml)	176 (104; 305)	566 (400; 746)	>0.000	0.83
MCP-1 (pg/ml)	449 (325; 583)	735 (515; 878)	0.001	0.78
suPAR (ng/ml)	3.5 (3.0; 3.9)	5.2 (3.6; 8.4)	0.002	0.78
MIG (pg/ml)	100 (72; 122)	152 (110; 308)	0.010	0.75
HGF (ng/ml)	3.69 (2.31; 4.91)	5.58 (3.55; 9.76)	0.034	0.71
Insulin (ng/ml)	1.14 (0.89; 2.60)	2.35 (1.09; 6.94)	0.083	0.66
IL-6 (pg/ml)	4.7 (3.6; 15.0)	11.7 (4.7; 33.8)	0.084	0.66
IL-1 β (pg/ml)	3.9 (1.4; 7.4)	2.5 (0.9; 4.9)	0.307	0.59
Leptin (ng/ml)	44.2 (32.1; 71.2)	60.2 (34.1; 91.3)	0.361	0.57
NGF (pg/ml)	81.8 (42.1; 302.5)	98.3 (67.8; 176.0)	0.668	0.54

^a Mann Whitney test for equality

^b Area under the receiver operating characteristic curve

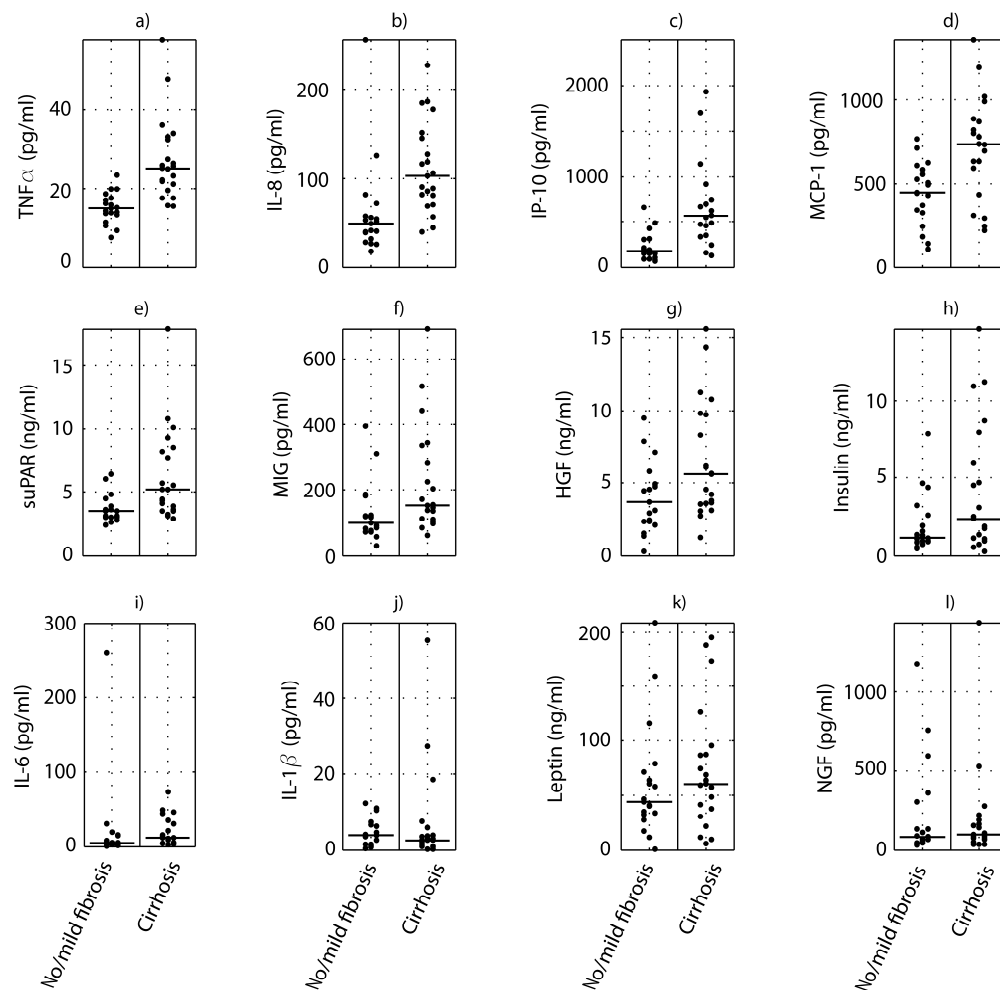


Figure 1 Plasma concentrations of fibrosis markers with medians shown as horizontal lines. The plots show a) TNF- α (tumor necrosis factor- α), b) IL-8 (interleukin 8), c) IP-10 (interferon- γ inducible protein 10), d) MCP-1 (monocyte chemotactic protein-1), e) suPAR (soluble urokinase-type plasminogen activator), f) MIG (monokine induced by γ -interferon), g) HGF (human hepatocyte growth factor), h) insulin, i) IL-6 (interleukin 6), j) IL-2 β (interleukin 2- β), k) leptin and (l) NGF (nerve growth factor). For each of c), i), and j), one outlier is not shown but has been included in the median determination.

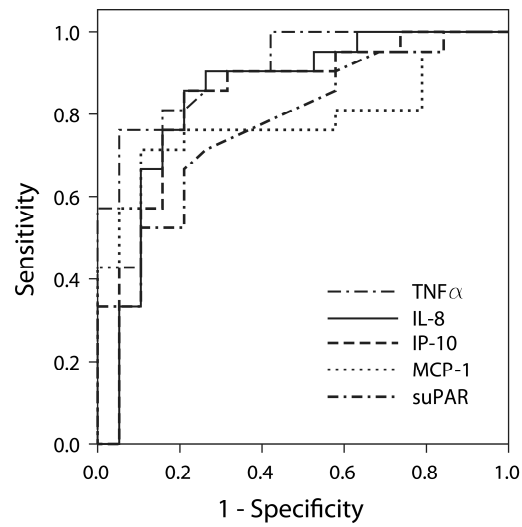


Figure 2 ROC (receiver operating characteristic) curves comparing the ability of TNF- α (tumor necrosis factor- α), IL-8 (interleukin 8), IP-10 (interferon- γ inducible protein 10), MCP-1 (monocyte chemotactic protein-1), suPAR (soluble urokinase-type plasminogen activator) to discriminate between cirrhosis and no/mild fibrosis in patients with chronic hepatitis C, genotype 1.

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