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Full-length article

Title: Soluble urokinase plasminogen activator receptor levels are associated with severity of fibrosis in non-alcoholic fatty liver disease

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Abbreviations

APRI = AST-to-platelet ratio index; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AUC = area under the curve; CRP = C-reactive protein; D_I = domain I; D_{II} = domain II; D_{III} = domain III; ELISA = enzyme-linked immunosorbent assay; GUCI = Göteborg University Cirrhosis Index; HCV = hepatitis C virus infection; HC = healthy controls; HSC = hepatic stellate cells; NAFLD = non-alcoholic fatty liver disease; NAS = NAFLD activity score; SLE = systemic lupus erythematosus; suPAR = soluble urokinase plasminogen activator receptor; uPAR = urokinase plasminogen activator receptor

Abstract

The identification of individuals with severe liver fibrosis among patients with chronic liver disease is of major importance when evaluating prognosis, potential risk for complications, and when deciding treatment strategies. Although percutaneous liver biopsy is still considered “gold standard” for staging of liver fibrosis, attempts to find reliable non-invasive markers of liver fibrosis are frequent. Inflammation is essential for the progression of fibrosis. The urokinase plasminogen activator (uPA) and its receptor (uPAR) have been associated with hepatic inflammation and fibrosis in mice. High serum concentrations of soluble uPAR (suPAR) are suggested to be involved in inflammation, tissue remodeling and cancer metastasis. Here, we evaluated serum suPAR as a non-invasive test to detect liver fibrosis in 82 well-characterized non-alcoholic fatty liver disease (NAFLD) patients, and in 38 untreated patients with chronic hepatitis C (HCV) infection at the time of their first liver biopsy. suPAR levels were increased in chronic liver disease compared to blood donors ($p<0.001$). HCV patients had higher suPAR concentrations than NAFLD patients ($p<0.002$). suPAR levels were associated with the severity of fibrosis, particularly in NAFLD, but did not correlate with inflammation. Regarding the performance in predicting severity of fibrosis, suPAR was essentially as good as other commonly used non-invasive fibrosis scoring systems. The results in HCV confirm previous observations. However, this is the first study to investigate suPAR as a biomarker in NAFLD and the results indicate that suPAR may constitute a severity marker related to fibrosis and prognosis rather than reflecting inflammation.

Introduction

Hepatic fibrogenesis is a maladaptive wound-healing process that occurs in response to persistent, injurious stimuli affecting the hepatocytes. This results in a stereotypical chronic inflammatory response leading to hepatic stellate cell (HSC) activation that produces an accumulation of extracellular matrix complexes.¹ The crosslinking of collagen fibrils within the extracellular matrix leads to fibrous scar formation and eventual distortion of the hepatic architecture.¹ The presence and stage of liver fibrosis is of paramount importance in patients with chronic liver disease when deciding treatment strategies, response to therapy, prognosis, and the potential risk for complications.

The “gold standard” for staging liver fibrosis is still percutaneous liver biopsy.² However, important limitations to biopsy exist. Significant complications, requiring hospital admission or prolonged hospital stay, occur in 1–5% of patients, and mortality has been reported to range between 0.1–1 in 1000 patients.³ In addition, liver biopsy has two major limitations: sampling error and inter-observer variability. A needle liver biopsy only represents 1:50 000 of the total organ, *i.e.* the potential for sampling error remains substantial. Autopsy and laparoscopy studies have demonstrated that cirrhosis is missed on a single blind liver biopsy in between 10–30% of cases.^{4,5} The intra- and inter-observer variability may range from 15% to as high as 33% for determining fibrosis stage.⁵

The attempts to find reliable non-invasive markers of liver fibrosis have recently increased dramatically as a result of the evolving novel therapies for hepatitis C and B.^{6,7} By analogy, a considerable interest has arisen in extending this work into the field of

non-alcoholic fatty liver disease (NAFLD) because of its increased prevalence.^{8,9} Moreover, recent long term studies suggest that development of fibrosis in NAFLD has an important prognostic significance.¹⁰

The soluble urokinase plasminogen activator receptor (suPAR) constitutes the circulating form of the glycosylphosphatidylinositol-linked membrane protein urokinase-type plasminogen activator receptor (uPAR; CD87), and is involved in inflammation, tissue remodeling and cancer metastasis.^{11,12} uPAR is expressed by a wide range of immune cells whereas suPAR can be detected in different body fluids, including serum and plasma.¹¹⁻¹⁷ Cell-surface uPAR expression is up-regulated upon stimulation with growth factors and cytokines such as interleukin-1 β and tumor necrosis factor.^{18,19} The full length suPAR shed from the cell surface contains three domains (D_{I-III}), and suPAR can occur in different cleaved forms consisting of only D_I or D_{II-III}, with partly divergent biological functions.^{12,20} Increased levels of suPAR have been found to predict disease outcome in various forms of cancer and infectious diseases.^{21,22} Furthermore, suPAR blood levels correlate with markers of organ dysfunction such as creatinine, urea, cystatin C, bilirubin and albumin in the severely ill.²¹ In chronic liver disease with progressive liver fibrosis/cirrhosis, suPAR has also been shown to be elevated and correlate with poor prognosis.²³⁻²⁵ In patients with systemic lupus erythematosus (SLE), suPAR seems to reflect organ damage/disease severity rather than ongoing inflammation.²⁶

The main aim of the present study was to evaluate serum suPAR as a non-invasive test to detect liver fibrosis in patients with NAFLD and chronic hepatitis C virus infection (HCV), respectively. The secondary aim was to compare the diagnostic performance of

suPAR with a number of simple non-invasive tests previously reported to be of clinical value in identifying advanced liver fibrosis.

Methods

Patients and controls

The basis for this investigation is formed by patients referred for evaluation to the Department of Gastroenterology and Hepatology or to the Department of Infectious Diseases at Linköping University Hospital because of persistently (>6 months) elevated serum aminotransferases and/or alkaline phosphatase. A diagnostic work-up was performed including physical examination, laboratory investigations and liver biopsy to identify toxic, metabolic, viral and autoimmune liver diseases. Relevant clinical and laboratory data were collected at the time of liver biopsy. 82 patients who reported average weekly alcohol consumption <140 g were diagnosed with biopsy-proven hepatic steatosis without any other concomitant liver disease or medication associated with fatty infiltration of the liver. These subjects constitute the NAFLD study group. 38 untreated patients who underwent liver biopsy were diagnosed with HCV based on serologic and genotypic findings and were subsequently included in the HCV study group.

In addition, 100 blood donors (50 men, 50 women; mean age 45.8 years; range 22–70) without ongoing medication served as healthy controls (HC) for the suPAR analyses.

Subjects with NAFLD and HCV had blood drawn immediately prior to liver biopsy. Sera were kept frozen at –70°C until analysis.

Histopathology

Liver biopsies of all subjects were performed percutaneously with ultrasound guidance and 1.6 mm Biopince needles on an outpatient basis. Global inflammation grade (0–4) and fibrosis stage (0–4) was assessed according to the standardized semi-quantitative histological scoring system developed by Batts and Ludwig.²⁷ Portal inflammation was reported separately (0–4) as described by Knodell *et al.*²⁸ Histopathological activity in NAFLD was assessed with the NAFLD activity score (NAS), which is the unweighted sum of steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2),²⁹ and steatosis was also assessed quantitatively as described by Franzén *et al.*³⁰

Scoring of fibrosis

In Table II, the 4 non-invasive fibrosis scoring systems that were used in the study are presented; the NAFLD fibrosis score, the AST-to-platelet ratio index (APRI), the BARD score and the Göteborg University Cirrhosis Index (GUCI) (Table II).^{31–34}

Routine laboratory measures

All biochemical assessments were performed in routine at the Department of Clinical Chemistry, Linköping University Hospital and included albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), high-sensitivity C-reactive protein (hsCRP), γ -glutamyl transferase (GGT), bilirubin, prothrombin complex international normalized ratio (PK-INR), and platelet count. Furthermore, in the NAFLD patients fasting plasma glucose, and fasting serum insulin was analyzed. Insulin resistance was assessed by the quantitative insulin sensitivity check index (QUICKI; $[1/(\log(\text{fasting insulin } \mu\text{U/mL}) + \log(\text{fasting glucose mg/dL}))]$).³⁵

The presence of metabolic syndrome and body mass index (BMI) were recorded at blood sampling (body weight [kg]/m²).³⁶

Serotyping, RNA detection/quantification, and genotyping

The anti-HCV Microparticle Enzyme ImmunoAssay (Abbott, Abbott Park, IL, USA) was used for HCV serology screening. Confirmation was performed with a recombinant immunoblot assay. RNA was extracted from plasma and initially analyzed with an in-house nested polymerase chain reaction, followed by the Cobas Amplicor Hepatitis C virus test version 2.0 (Roche Diagnostics, Branchburg, NJ, USA). HCV-RNA was quantified by the branched DNA technique (Quantiplex HCV RNA version 2.0; Chiron Corp., Emeryville, CA, USA). HCV genotypes were determined after hybridization of biotin-labeled polymerase chain reaction products to oligonucleotide probes bound in strips on nitrocellulose membranes (Inno-Lipa, Innogenetics, Brussels, Belgium). All analyses were performed at Linköping University Hospital except for HCV genotyping, which was performed at the Department of Microbiology, Karolinska University Hospital, Stockholm, Sweden.

suPAR assay

Sera were analyzed in duplicate for suPAR levels using suPARnostic® standard ELISA from Virogates (Birkerød, Denmark). Briefly, serum samples, standard and curve control samples were mixed with peroxidase-conjugated detection antibody and transferred to pre-coated plates. Following 1h incubation in room temperature (RT) the wells were washed and TMB substrate added. The plates were incubated for 20 min at RT, stop solution was added and the plates read at 450 nm (Sunrise™ microplate reader with Magellan™ software, Tecan, Grödig, Austria).²⁶

Statistics

Differences in suPAR levels between groups were analyzed using ANOVA followed by the post-hoc test Tukey. Differences between groups of diagnoses were analyzed with T-test or Chi-2, were appropriate. Correlation analyses were performed using Pearson's correlation test. Receiver-operating characteristics (ROC) curves were constructed to assess the overall accuracy of suPAR and non-invasive fibrosis scoring systems to discriminate between fibrosis stage 0–2 and fibrosis stage 3–4. The ROC curve is a plot of sensitivity vs. specificity (1 – specificity) for all possible cut-off values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values close to 1.0 indicating a high diagnostic accuracy.

A 2-tailed *p* value of less than 0.05 was considered significant. All statistical analyses were performed with the SPSS Statistics 19–20 (IBM, Armonk, NY, USA) software. GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA) was used for figures.

Ethics

The study protocol was approved by the Linköping University Ethical Review Board (2010/406–31 and 02–454).

Results

Patient data

In total, 82 individuals with NAFLD and 38 with HCV were enrolled in the study. 20 (17%) patients had advanced fibrosis (stage 3-4); 16 of these were diagnosed with NAFLD and 4 with HCV. The remaining 62 patients had no or mild fibrosis (stage 0-2). The age of the HC was similar to that of the HCV patients, whereas NAFLD patients were significantly older. Female gender was similar among HC and HCV patients, whereas NAFLD patients were males to a higher extent than HC. Characteristics of all patients and controls are given in Table I.

Increased suPAR levels in patients with chronic liver disease

As demonstrated in Figure 1a, suPAR levels were significantly higher in NAFLD patients compared with HC (4.8 ± 1.8 vs. 3.9 ± 0.9 ; $p < 0.001$); a similar difference was found between HCV patients and HC (5.9 ± 2.1 vs. 3.9 ± 0.9 ; $p < 0.001$). In addition, HCV patients showed higher suPAR levels compared with NAFLD (5.9 ± 2.1 vs. 4.8 ± 1.8 ; $p < 0.002$). As indicated in Figure 1b, regardless of background disease advanced fibrosis (stage 3-4) was significantly associated with higher suPAR levels in comparison with patients with no or mild fibrosis (stage 0-2) (6.2 ± 1.9 vs. 5.0 ± 1.9 ; $p = 0.013$). When restricting the study population to NAFLD patients exclusively, as depicted in Figure 1c, individuals with advanced fibrosis still showed higher suPAR levels compared with those with no or mild fibrosis (5.7 ± 1.8 vs. 4.6 ± 1.7 ; $p = 0.025$). A corresponding trend towards higher suPAR was found for the 4 patients with advanced fibrosis relative to the others with HCV (7.8 ± 1.3 vs. 5.6 ± 2.1 ; $p = 0.050$) as demonstrated in Figure 1d. Neither inflammation grade according to Batts and Ludwig, nor portal inflammation as defined by Knodell were

associated with suPAR levels in the HCV group. Similarly, NAS was not associated with suPAR levels in the NAFLD group.

Association of suPAR with routine laboratory measures and histopathology

In NAFLD patients, suPAR levels were significantly associated with age, presence of diabetes mellitus, severity of fibrosis, PK-INR and QUICKI (Table III), but not with presence of metabolic syndrome, gender, BMI, hsCRP, ALP, AST, ALT, steatosis grade, NAS, or portal inflammation. In HCV patients, suPAR levels were associated with age, hsCRP, ALT, AST, ALP, but not with presence of diabetes mellitus, gender, BMI, PK-INR, fibrosis, steatosis grade or inflammation grade (Table III). suPAR levels did not significantly differ between HCV genotypes; and suPAR was not correlated with the amount of HCV-RNA.

suPAR versus severity of fibrosis

For all 120 patients, suPAR displayed an AUROC of 0.716 (Figure 2a) which was almost similar to the non-invasive fibrosis scoring systems, *i.e.* APRI (AUC 0.763) and GUCI (AUC 0.771). In NAFLD, an AUROC of 0.722 was achieved for suPAR. The corresponding figures for NAFLD fibrosis score, APRI, BARD, and GUCI were 0.773, 0.842, 0.709, and 0.856, respectively (Figure 2b). In HCV patients, an AUROC of 0.848 was achieved for suPAR compared with APRI 0.720, and GUCI 0.720.

Discussion

In the past decade, many investigators have proposed non-invasive replacements to liver biopsy, including either a single biochemical marker or a combination of tests, both

serum markers and physical measurement of liver stiffness.^{1,4,7,8} Unfortunately, at the time of our study, transient elastography was unavailable at our center. While some investigators have focused on a combination of laboratory tests such as reversal of AST/ALT ratio or APRI, others have been searching for novel markers of fibrosis and inflammation.¹ Despite sharing the common pathway for fibrosis, an imbalance between synthesis and degradation of extracellular matrix by activated HSC, there are currently no liver specific markers available that could accurately reflect liver fibrosis of different etiologies.^{1,37}

Herein, suPAR was analyzed in two patient groups with chronic liver disease. We demonstrate that suPAR levels are associated with severity of fibrosis in both NAFLD and HCV. With only one exception (being hsCRP levels in HCV patients), inflammation – including biomarkers and histopathology – was not associated with suPAR levels. This is consistent with our previous finding for SLE patients where serum suPAR correlated strongly with organ damage rather than inflammatory activity.²⁶

The present study is the first to evaluate suPAR as a biomarker in NAFLD. The development of NAFLD is associated with insulin resistance, which is attributed to inflamed adipose tissue.¹⁰ In our well-characterized NAFLD cohort, we show that suPAR levels are higher in comparison with HC, whereas lower concentrations were found compared to HCV patients. In addition, we observed an association between suPAR levels and QUICKI as well as the presence of diabetes mellitus, whereas the association with metabolic syndrome did not reach statistical significance ($p=0.14$). Thus, a connection between suPAR and the degree of inflammation cannot be excluded in NAFLD. In line with this observation, it was recently shown that suPAR levels are

associated with incident diabetes among overweight but not obese individuals with impaired glucose regulation.³⁸ Lifestyle factors, such as smoking and physical activities, as well as age have an effect on baseline suPAR levels also in a healthy population.³⁹ As expected, age was associated with suPAR in both patient groups (Table III); this is important to take into account in the assessment of measurements in any patient group.

Although we here analyzed a limited number of HCV patients, suPAR was higher in patients with advanced compared to mild fibrosis (Figure 1b). Indeed, suPAR has previously been evaluated as a non-invasive fibrosis marker in HCV. Berres and colleagues concluded that serum suPAR has a high diagnostic specificity and sensitivity to differentiate mild (stage 1–2) from severe (stage 3–4) fibrosis with an AUROC of 0.774.²⁵ Zimmermann *et al* presented similar results and pointed out that suPAR levels above 9 ng/mL could constitute a strong predictor of mortality or the need for liver transplantation.²³ Thus, our results regarding HCV are clearly in line with previous observations. Considering the question raised by Dr Filik, we did not find any correlation between viral load and suPAR levels.⁴⁰ Thus, it is possible that suPAR could be attributed to liver fibrosis *per se* rather than due to the viral infection.

We found a significant moderate correlation between suPAR concentration and fibrosis stage, as well as a difference between mild and advanced fibrosis (Figure 1b). The NAFLD fibrosis score, APRI, BARD and GUCI are all commonly used scores for estimating the severity of fibrosis.⁴¹ APRI and GUCI were primarily developed for HCV. Herein, these scores were compared with suPAR alone regarding performance in predicting severity of fibrosis. Intriguingly, for all 120 patients, suPAR was essentially as useful as APRI and GUCI. In the 82 NAFLD patients, NAFLD fibrosis score, APRI and GUCI

performed better than suPAR, but suPAR was at least as good as BARD. The question whether suPAR gives additional information regarding severity of fibrosis was beyond the scope of this paper. One possibility to further investigate this would however be to create a fibrosis score where suPAR is included as a separate component and apply this to larger study populations. One advantage by analyzing suPAR is the stability of the molecule as it is not substantially affected by freeze/thaw cycles. In addition, the inter-day variations are negligible and the concentration is not influenced by dietary intake.⁴²

Whether suPAR constitutes an epiphenomenon or if it plays an active role in the development of liver fibrosis remains open. In the liver, activation of the innate immune system has a critical role in maintaining homeostasis and liver regeneration, as well as disease pathogenesis, acting in a cooperative rather than independent fashion.⁴³ To our knowledge, there are no published data on how suPAR affects the activation of HSC and potential effects on the balance between synthesis and degradation of extracellular matrix. However, Koch *et al* recently demonstrated that the human HSC line LX2 has the capability to spontaneously secrete suPAR during cultivation although the levels were low.⁴⁴ In addition, HSC from healthy donors have been shown to produce uPAR, and the addition of uPA enhanced the chemotactic and chemoinvasive properties of the HSC.⁴⁵ The role of innate immune components such as mast cells, neutrophils, macrophages, NK cells, complement pathway proteins, secreted pattern-recognition receptors, and acute phase reactants have been implicated in liver disease.^{46,47} To induce inflammation and subsequent organ damage, these cells need to cross the blood barrier and be directed to the tissue. The classical chemoattractant for neutrophils is interleukin-8.⁴⁸ However, it has been shown that suPAR fragment D_{II-III} can act as a chemoattractant for neutrophils and monocytes.⁴⁹⁻⁵¹ Neutrophils and monocytes are also important for the

release of suPAR, which could induce further recruitment of inflammatory cells and perpetuate the inflammation into a vicious circle.^{44,52}

To conclude, this is the first study to describe an association between circulating suPAR and severity of fibrosis in NAFLD. In addition, we confirmed previous reports on HCV demonstrating a corresponding association. Accordingly, suPAR appears to reflect severity of fibrosis rather than inflammation in these conditions, and thus constitute a promising biomarker candidate.⁵³ Further prospective studies will have to address the question if suPAR provides additional information compared to the present non-invasive scoring systems for liver fibrosis.

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Disclosure statement: Nothing to declare.

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Legends

Figure 1: Serum suPAR concentrations in NAFLD and HCV patients, and healthy controls (HC) analyzed by ELISA (a). *** $p < 0.001$; ** $p < 0.002$. Serum suPAR concentrations according to fibrosis stage; mild (stage 0–2) and advanced (stage 3–4), demonstrated for all patients (b), NAFLD patients only (c) and HCV patients only (d). Open circles represent NAFLD and filled circles HCV. Mean concentrations $\pm$ SD are indicated by horizontal bars.

Figure 2: Serum soluble urokinase plasminogen activator receptor (suPAR) concentration as a predictor of severity of fibrosis in comparison with other commonly used non-invasive fibrosis scoring systems in all 120 patients (a), and in the 82 NAFLD patients exclusively (b). Area under the ROC curve (AUROC) values are given in the main text.

Table I. Characteristics of the non-alcoholic fatty liver disease (NAFLD) and hepatitis C virus (HCV) infected study populations, as well as the healthy controls (HC).

	NAFLD	HCV	HC	
Patient data	(n=82)	(n=38)	(n=100)	<i>p</i> =
Age (years)	59.8 ± 11.7	45.3 ± 15.8	45.8 ± 11.9	<0.001
Gender (male)	55 (67%)	22 (58%)	50 (50%)	0.02
BMI	28.9 ± 4.4	24.9 ± 4.1	*	0.025
Diabetes mellitus	42 (51%)	1 (2.6%)	0 (0%)	<0.001
Platelet count (10 ⁹ /L)	231 ± 66	247 ± 74	*	0.24
Albumin (g/L)	42 ± 3.8	41 ± 3.8	*	0.13
hsCRP (mg/L)	3.3 ± 5.0	1.3 ± 1.9	*	0.021
Bilirubin (μmol/L)	13 ± 5.4	15 ± 23	*	0.62
ALP (μkat/L)	3.2 ± 2.2	2.4 ± 1.4	*	0.057
AST (μkat/L)	0.65 ± 0.28	1.6 ± 2.6	*	0.001
ALT (μkat/L)	1.1 ± 0.67	2.6 ± 3.2	*	<0.001
GGT (μkat/L)	1.3 ± 1.2	1.2 ± 1.3	*	0.72
PK-INR	1.0 ± 0.09	1.0 ± 0.09	*	0.31

Differences between groups of diagnoses were analyzed with T-test or Chi-2 were appropriate.

* Not analyzed

Table II. The following formulas were used for calculation of the fibrosis scoring systems.

NAFLD fibrosis score ³¹	$-1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{Impaired fasting glucose/Diabetes (yes=1, no=0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{Platelet count (10}^9\text{/L)} - 0.66 \times \text{albumin (g/dL)}$
APRI ³²	$\text{AST level /AST}_{\text{ULN}} \times 100 / \text{Platelet count (10}^9\text{/L)}$
BARD-score ³³	$\text{BMI} \geq 28 = 1 \text{ p, AST/ALT ratio} \geq 0.8 = 2 \text{ p, Diabetes} = 1 \text{p}$
GUCI ³⁴	$\text{AST (}\mu\text{kat/L)} / \text{AST}_{\text{ULN}} (\mu\text{kat/L)} \times \text{prothrombin-INR} \times 100 / \text{Platelet count (10}^9\text{/L)}$

ULN = Upper limit of normal

1 $\mu\text{kat/l}$ = 60 U/L

Table III. Correlations between serum suPAR levels and disease variables using Pearson.

Factor \ Disease	NAFLD		HCV	
	<i>r</i> =	<i>p</i> =	<i>r</i> =	<i>p</i> =
Age (years)	0.43	<0.0001	0.39	0.017
BMI		NS		NS
hsCRP		NS	0.36	0.027
ALP		NS	0.35	0.041
AST		NS	0.37	0.023
ALT		NS	0.36	0.026
PK-INR	0.24	0.030		NS
HCV-RNA		*		NS
QUICKI	−0.32	0.016		*
Fibrosis stage	0.29	0.008		NS
Inflammation grade		NS		NS
NAS		NS		*
Steatosis (quantitative)		NS		*

* Not analyzed

NS = not significant