Research paper

A PDGFRβ-based score predicts significant liver fibrosis in patients with chronic alcohol abuse, NAFLD and viral liver disease

Joeri Lambrecht a, Stefaan Verhulst a, Inge Mannaerts a, Jan-Peter Sowa c, Jan Best c, Ali Canbay c, Hendrik Reynaert a,b, Leo A. van Grunsven a,*

a Department of Basic (Bio-)Medical Sciences, Liver Cell Biology Research Group, Vrije Universiteit Brussel, Brussels, Belgium
b Department of Gastroenterology and Hepatology, University Hospital Brussels (UZBrussel), Brussels, Belgium
c Department of Gastroenterology, Hepatology and Infectious Diseases, University Hospital Magdeburg, Magdeburg, Germany

ARTICLE INFO

Article history:
Received 28 February 2019
Received in revised form 18 April 2019
Accepted 18 April 2019
Available online 27 April 2019

Keywords:
Platelet derived growth factor receptor
Biomarker
Extracellular vesicle
Diagnosis
Hepatic stellate cell

A B S T R A C T

Background: Platelet Derived Growth Factor Receptor beta (PDGFRβ) has been associated to hepatic stellate cell activation and has been the target of multiple therapeutic studies. However, little is known concerning its use as a diagnostic agent.

Methods: Circulating PDGFRβ levels were analysed in a cohort of patients with liver fibrosis/cirrhosis due to chronic alcohol abuse, viral hepatitis, or non-alcoholic fatty liver disease (NAFLD). The diagnostic performance of PDGFRβ as individual blood parameter, or in combination with other metabolic factors was evaluated.

Findings: sPDGFRβ levels are progressively increased with increasing fibrosis stage and the largest difference was observed in patients with significant fibrosis, compared to no or mild fibrosis. The accuracy of sPDGFRβ-levels predicting fibrosis could be increased by combining it with albumin levels and platelet counts into a novel diagnostic algorithm, the PRTA-score, generating a predictive value superior to Fib-4, APRI, and AST/ALT. The sPDGFRβ levels and the PRTA-score are independent of liver disease aetiology, thus overcoming one of the major weaknesses of current non-invasive clinical and experimental scores. Finally, we confirmed the diagnostic value of sPDGFRβ levels and the PRTA-score in an independent patient cohort with NAFLD which was staged for fibrosis by liver biopsy.

Interpretation: The PRTA-score is an accurate tool for detecting significant liver fibrosis in a broad range of liver disease aetiologies.

Fund: Vrije Universiteit Brussel, the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWV-Flanders) (HILIM-3D; SBO140045), and the Fund of Scientific Research Flanders (FWO).

© 2019 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The chronic presence of liver-injury causing agents, including alcohol, Hepatitis B or C virus (HBV/HCV) infection, and non-alcoholic steatohepatitis/fatty liver disease (NASH/NAFLD), leads to the activation of hepatic stellate cells (HSCs) toward a myofibroblastic phenotype [1]. This activation process is characterized by an excessive deposition of extracellular matrix, scar tissue formation, and an enhanced responsiveness of the HSCs toward various stimulating factors secreted by their microenvironment [2]. Such enhanced responsiveness is facilitated by an elevated expression of cell membrane receptors such as several tyrosine kinases receptors [2]. One such receptor is the platelet derived growth factor receptor (PDGFR), of which 2 variants can be found: PDGFR-alpha (PDGFRα) which is constitutively expressed by HSCs, and PDGFR-beta (PDGFRβ) whose expression increases during HSC activation [3]. Binding of the PDGF isoforms to their respective receptors induces receptor dimerization, phosphorylation of tyrosine residues at

Abbreviations: PDGFRβ, platelet derived growth factor receptor beta; sPDGFRβ, soluble pdgfrβ; AUC, area under the curve; PRTA-score, Spdgfrβ-thrombocyte albumin score; Fib-4, fibrosis-4; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBV, Hepatitis B virus; HCV, Hepatitis C virus; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; HSC, hepatic stellate cell; PDGF-Rx, platelet derived growth factor receptor alpha; ARFI, acoustic radiation force impulse; MRE, magnetic resonance elastography; ELF, enhanced liver fibrosis; IU/L, International units per litre; ULN, upper limit of normal; NPI, non-parenchymal fraction; FACS, fluorescence-activated cell sorting; CCL, carbon tetrachloride; PBS, phosphate buffered saline; EV, extracellular vesicle; NTA, nanoparticle tracking analysis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; αsMA, alpha smooth muscle actin; HSP70, heat shock protein 70; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ROC, receiver operating characteristics; BMI, body mass index; ECM, extracellular matrix; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; RNA, ribonucleic acid; WB, Western blot; n, number; IQR, interquartile range; ALD, alcoholic liver disease.

* Corresponding author at: Vrije Universiteit Brussel, Faculty of Medicine and Pharmacy, Department of Basic (Bio-)Medical Sciences, Liver Cell Biology Research Group, Laarbeeklaan 103, 1090 Brussels (Jette), Belgium.
E-mail address: lvgrunsv@vub.be (L.A. van Grunsven).
the intracellular domain of the receptor, and activation of various pro-fibrogenic cascades [2,4].

Diagnosis of liver fibrosis onset and progression remains an important issue in the current clinical settings. The gold standard, and the diagnostic tool with highest specificity and sensitivity, remains the liver biopsy, an invasive procedure associated with multiple drawbacks including inter-and intra-observer variability [5], discomfort for the patient such as pain and post-procedure complications [6], and a questionable cost-benefit ratio [7]. In order to overcome these drawbacks, multiple non-invasive diagnostic tools have been proposed [8]. Imaging modalities are a subgroup of such non-invasive diagnostic tools, measuring the elastic properties and stiffness of the liver tissue [9]. Transient elastography (such as FibroScan®) [10] and acoustic radiation force impulse (ARFI) [11] are examples which have been validated in various aetiologies of liver disease. Furthermore, the use of serological markers has been proposed for non-invasive assessment of liver fibrosis. The use of serum markers may rely on the detection of a single parameter, or a group of parameters combined into a diagnostic algorithm [8]. In the current clinical setting, several serological algorithms have gained popularity, such as the fibrosis 4 (Fib-4) score [12], enhanced liver fibrosis (ELF) test [13], aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio [14], and the AST to platelet ratio index (APRI) [15]. However, although the implementation of serological markers and imaging modalities has led to less liver biopsies, it has not yet resulted into its full redundancy. Avoidance of liver biopsy for staging of fibrosis will only be possible when a non-invasive marker has been found which is independent of liver disease aetiology, easily accessible, with low cost, and with high specificity and sensitivity for both early and late stages of liver fibrosis [16]. Especially the limited accuracy for diagnosis and progression of early stage liver fibrosis remains a weakness of current non-invasive diagnostic tools, which thus prevents an as early as possible therapeutic intervention (or life style change) to avoid fibrosis progression. As fibrosis has been shown to be the most important predictor of liver-related mortality [17], early intervention would significantly reduce mortality.

In this study, we present an analysis of circulating PDGFRα protein levels in a patient population with different aetiologies of liver fibrosis, being chronic alcohol abuse, chronic viral (HBV/HCV) infection and NAFLD. Additionally, we propose the implementation of soluble PDGFRα levels into a novel diagnostic algorithm, the sPDGFRα/thrombocyte albumin (PRTA)-score, which yields a high discriminative capacity for diagnosis of significant fibrosis.

2. Materials and methods

2.1. Animal studies

The use and care of animals was reviewed and approved by the Ethical Committee of Animal Experimentation of the Vrije Universiteit Brussel (VUB, Belgium) in project 16-212-2, and was carried out in accordance to European Guidelines for the Care and Use of Laboratory Animals. Mice were housed in a controlled environment with free access to chow and water. Quiescent hepatic stellate cells were isolated from male Balb/c mice (Charles River Laboratories, L’Arbresle, France) (25–30 weeks old) as described earlier [18]. Briefly, murine livers were perfused with enzymatic solutions, followed by low-speed centrifugation steps to remove hepatocytes. Hepatic stellate cells were purified from the non-parenchymal fraction based on their buoyancy, using an 8% Nycodez solution. Isolated HSCs were cultured on regular tissue culture dishes (Greiner Bio-One, Vilvoorde, Belgium), in Dulbecco’s modified Eagle’s medium (Lonza, Verviers, Belgium) supplemented with 10% exosome-depleted foetal bovine serum (System Biosciences, Mountain View, USA), 2 MmL-glutamine (Ultraglutamine, Lonza, Verviers, Belgium) and 1000 μg/mL penicillin and 100 μg/mL streptomycin (Pen-Strep®) (Lonza), inducing an in vitro myofibroblastic transdifferentiation.

The different liver cell populations were isolated based on cell-type specific protein expression, as described earlier [19]. Briefly, murine livers were perfused with enzymatic solutions, followed by low-speed centrifugation steps to separate the non-parenchymal fraction from the hepatocyte population. The non-parenchymal fraction (NPF) was incubated with anti-f4/80-APC (MF8021, Thermo Scientific, USA) and anti-CD32-PE (ab30357, Abcam, UK). NPF was then analysed with anti-CD32-PE (ab30357, Abcam, UK) and used to isolate liver sinusoidal endothelial cells (LSEC, CD32 + F4/80-UV-), Kupffer cells (CD32-FO/80 + UV+) and HSCs (CD32-F4/80-UV+) (Lonza), 100 U/ml penicillin and 100 μg/ml streptomycin (Pen-Strep®) (Lonza), inducing an in vitro myofibroblastic transdifferentiation.

For in vivo induction of liver fibrosis, 10-week old mice received 8 intraperitoneal injections of 15 μl carbon tetrachloride (CCL4) diluted in 85 μl mineral oil (Sigma-Aldrich, St. Louis, MO, USA) per 30 g bodyweight over a period of 4 weeks. Mice were sacrificed 24 h after the last injection.

2.2. Cell-derived extracellular vesicle (EV) isolation

Conditioned medium was collected from cultured mouse HSCs after 2 days or 10 days of culture (new medium was added every 2 days) and
cleared from cellular debris by centrifugation at 300 g for 5 min (4 °C) and 2500 g for 20 min (4 °C). Large vesicle-like contaminants were depleted by centrifugation at 10,000g for 30 min. The supernatant was further centrifuged at 100,000g for 2 h (4 °C) to pellet EVs, which were then washed once by resuspension in phosphate-buffered saline (PBS) followed by a final ultracentrifugation step at 100,000g for 2 h (4 °C). The final EV pellet was resuspended in a small volume of PBS and characterized by use of the ZetaView® PMX110 (Particle Metrix, Meerbusch, Germany) which is equipped with nanoparticle tracking analysis (NTA) software, for particle size, zeta-potential, and concentration. The instrument was calibrated using 100 nm sized polystyrene particles and handled following manufacturer’s protocol. NTA measurements were executed at 11 different positions at a constant temperature of 23 °C.

2.3. Initial patient cohort

Patients were recruited from the Department of Gastroenterology of the University Hospital of Brussels (UZ Brussel), Belgium. The study protocol was approved by the local ethical committee of the UZ Brussel and Vrije Universiteit Brussel (reference number 2015/297; B.U.N. 143201525482) and was in accordance with the Declaration of Helsinki. Patients with alcohol abuse, chronic viral hepatitis and NAFLD were recruited. A healthy population that had no evidence of liver disease was recruited as control group. All participants signed an informed consent prior to inclusion to the study. Inclusion criteria for fibrosis and cirrhosis patients included: i) diagnosis confirmed by elastography; ii) availability of complete clinical information. Exclusion criteria were: i) HIV co-infection; ii) missing data on important variables; iii) idiopathic/un-identified origin of liver disease. Diagnosis of liver fibrosis and cirrhosis was based on physical examination, blood tests, and elastography techniques. Patients with viral or alcoholic liver disease underwent transient elastography (FibroScan®, Echosens, France). Patients with at least 10 valid liver stiffness measurements with a success rate of at least 60% were included in the final analysis. Cut-off values used to discriminate fibrotic stages equal or more than F2, F3 or F4, were respectively 7.2 kPa, 9.5 kPa and 12.5 kPa [20]. Acoustic radiation force impulse (ARFI) was applied to determine the stage of liver fibrosis in those patients with NAFLD. Cut-off values of 1.25 m/s, 1.54 m/s and 1.84 m/s were used to identify a fibrotic stage equal to, or more than F2, F3, and F4 respectively.

2.4. Blood collection

Blood samples were collected by venepuncture into evacuated EDTA-KE S-Monovette tubes (Sarstedt AG & Co, Nümbrecht, Germany) on the day of liver biopsy or elastography. Blood specimens were subjected to haematological and biochemical analyses. Plasma was created within maximum 2 h after collection, using a two-step centrifugation protocol consisting of 1500g for 10 min (4 °C) and 2000g for 3 min (4 °C). Plasma was frozen at −80 °C until use.

2.5. Serological tests

Validation of the suggested fibrosis scoring was obtained by haematological analysis and diagnostic algorithms such as the NAFLD fibrosis score, Fib-4, APRI, and AST/ALT ratio. Fib-4 and APRI were calculated using following formulae:

\[
\text{FIB} = \text{age} \times \text{AST} \frac{\text{IU/L}}{\left(\text{platelet count} \times 10^9/\text{L}\right)} \times \left(\frac{\text{ALT} \text{IU/L}}{\text{ULN}}\right)^{1/2}
\]

\[
\text{APRI} = \frac{\text{AST} \text{IU/L}}{\text{ULN}} \times \text{platelet count} \times 10^9/\text{L}
\]

2.6. Human liver tissue

Human liver tissue (Supplementary Table 1) was obtained from surgical procedures performed at the Department of Thoracic and Transplantation Surgery and Surgical Oncology of the University Hospital of Brussels (UZ Brussel), Belgium. Ethical approval was obtained from the local ethical committee of the UZ Brussel (Reference number 2015/278; B.U.N. 143201525406) and was in accordance with the Declaration of Helsinki. All participants signed an informed consent prior to inclusion to the study.

2.7. Validation patient cohort

For validation, 57 NAFLD-patients were recruited from a cohort initially recruited at the Alfred-Krupp-Krankenhaus Essen, Department for General and Visceral Surgery, Germany. The study protocol was approved by the institutional review board of the University Hospital Essen (Ethik-Kommission der Medizinischen Fakultät der Universität Duisburg-Essen; Germany; reference number 15-6356-BO) and was in accordance with the Declaration of Helsinki. All procedures adhered to the Declaration of Helsinki and the requirements of the IRB. Due to the retrospective nature of the validation study the IRB waived the need for written informed consent.

Patients of this cohort received bariatric surgery for weight reduction due to morbid obesity. Patients were eligible for the validation study, when liver histology, including fibrosis scoring, and a sufficient amount of serum was available. Patients received dietary and exercise counselling for 6 months prior surgery, without calorie restriction. A blood sample was collected for assessment of serum derived factors on the day of surgery (prior surgery) and liver tissue was sampled during bariatric surgery as a wedge biopsy. All data shown were recorded on the day of surgery.

Histological assessment of the liver tissue (steatosis, ballooning, lobular inflammation and fibrosis) was performed by two expert pathologists on HE- and Masson’s Trichrome stained 4 µm slides for each sample according to Kleiner et al. [21] and Desmet et al. [22].

2.8. Human hepatic stellate cells

Primary human HSCs were purchased from ScienCell (San Diego, USA), and used before passage 8 was reached. The LX-2 cell line, an immortalized human HSC line, was kindly provided by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, USA). Human HSCs were cultured in Dulbecco’s modified Eagle’s medium (Lonza) supplemented with 10% exosome-depleted foetal bovine serum (System Biosciences), 2 mM L-glutamine (Ultralglutamine 1®) (Lonza), 100 U/ml penicillin and 100 µg/ml streptomycin (Pen-Strip®) (Lonza).

2.9. Enzyme-linked immunosorbent assays (ELISA)

Human soluble PDGFRβ was measured with a commercially available ELISA kit (ThermoFisher scientific), according to manufacturer’s instructions. All plasma samples were diluted 1/10 with diluent provided by the manufacturer. Absorbance values were obtained with an iMark™ microplate absorbance reader (Bio-rad).

2.10. Statistical analysis

Data was analysed using GraphPad Prism 6 (GraphPad, Palo Alto, USA) statistical software. Quantitative variables are expressed as means ± standard deviation (SD) or expressed as box-plots (min to max). Statistical analyses were performed using the Student’s t-test, Mann-Whitney test, and Kruskal-Wallis test with Dunn’s post hoc test, as appropriate. Categorical variables were analysed using the Chi-square test. The baseline characteristics of the three patient cohorts were compared using the Chi-square test or Kruskal-Wallis test. To
determine the diagnostic accuracy and performance, receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was calculated. In order to identify ideal cut-off values, the Youden's index was calculated [23], and the sensitivity and specificity were computed. Correlation studies were executed using the Spearman's correlation test. The sufficiency of the sample size was confirmed by MedCalc version 18 (MedCalc Software, Ostend, Belgium) using in house preliminary results and a type I error rate (α) of 5% and a power (1-β) of 80%. Differences of obtained results were considered significant at p < 0.05.

3. Results

3.1. Human and murine aHSC-derived EVs are positive for PDGFRβ

We previously reported on the enhanced expression of PDGFRβ on extracellular vesicles (EVs) extracted from the plasma of chronic Hepatitis B or C virus (HBV/HCV)-infected patients with early (F ≤ 2) liver fibrosis [18]. This led us to speculate that these circulating EVs could represent the presence of activated HSCs in the injured liver. To investigate this hypothesis, we first verified the possible HSC-derived origin of these PDGFRβ-positive EVs. Vesicles extracted by ultracentrifugation from the culture medium of primary mouse- and human HSCs show a mean value ± SD.

3.2. PDGFRβ is up-regulated in the murine CCL4-injury model

We next analysed the expression of PDGFRβ in a well-established murine model of liver fibrosis, being repeated injections of carbon tetrachloride (CCL4), in which chronic necro-inflammatory damage leads to a significant activation of HSCs [27]. Protein analysis of the livers of 4-week CCL4-treated mice shows a significant up-regulation of PDGFRβ, both by western blot (Fig. 2a) and on staining (Fig. 2b), as compared to their healthy controls. RNA expression analysis shows a dominant expression of PDGFRβ in HSCs when compared to the non-parenchymal fraction (NPF), and to freshly isolated individual liver cell types being hepatocytes, liver sinusoidal endothelial cells, and Kupffer cells (Fig. 2c). This HSC-association of PDGFRβ is further illustrated by the correlation and overlap (Spearman's correlation coefficient (r) = 0.7838) in protein expression of PDGFRβ and alpha smooth muscle actin (αSMA), a marker for activated HSCs, in livers of CCL4-treated mice (Fig. 2d).

3.3. Clinical characteristics of the initial study population

Due to the several drawbacks of vesicle research such as the time-consuming and low-throughput character of current EV isolation techniques, the clinical setting is currently not ready to use EVs, nor their protein content, as biomarkers for the diagnosis of disease onset or its progression [8]. We therefore investigated the possibility to use total circulating PDGFRβ content as biomarker for liver fibrosis progression. To this end, the plasma PDGFRβ-content of a cohort of 148 patients and 14 healthy volunteers was analysed. Patients with various aetiologies of liver disease were included, being chronic alcohol abuse (n = 35), chronic HBV/HCV infection (n = 46), and NAFLD (n = 67) (Table 1). As expected, a significantly higher Body Mass Index (BMI) value is observed in patients with NAFLD.

Patients with alcoholic or viral liver disease underwent transient elastography measurements by FibroScan® to determine the stage of fibrosis/cirrhosis. Patients with NAFLD all suffered from Diabetes Mellitus type 2 and underwent ARFI to evaluate the degree of liver fibrosis/cirrhosis. In the total patient cohort, stage of liver fibrosis was distributed as follows: F0–1, n = 51 (34.46%); F2, n = 29 (19.59%); F3, n = 28 (18.92%); and F4, n = 40 (27.03%). Various fibrosis scoring algorithms, including the aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio, AST to platelet ratio index (APRI), and Fibrosis-4 (Fib-4) index, were calculated to further validate the early or late disease-character of the included patients (Supplementary Table 2).

3.4. sPDGFRβ predicts the presence of significant (F ≥ 2) fibrosis

Analysis of soluble PDGFRβ (sPDGFRβ) levels in our total patient cohort by ELISA identified an overall increase of sPDGFRβ according to the stage of liver fibrosis (Fig. 3a). Creating various sub-populations based on staging of fibrosis identified a significant discriminative value of sPDGFRβ levels to distinguish patients with significant fibrosis (F ≥ 2) from those with no or minimal fibrosis (F0–1); (median (25th: 75th percentile)): 9317 [6625;12,333] pg/mL vs 5581 [3838;10,069] pg/mL, respectively, p < 0.0001. Use of sPDGFRβ to predict the presence of significant fibrosis (F ≥ 2) was assessed by construction of AUROC, and generated an AUC of 0.7303 (95% confidence interval (CI): 0.6395–0.8211) (Table 2), which is considerably higher than the AUCs obtained from clinical scores such as Fib-4, APRI and AST/ALT, respectively 0.6635 (95% CI: 0.5690–0.7581), 0.6309 (95% CI: 0.5331–0.7286), and 0.5976 (95% CI, 0.4952–0.7001) (Table 2).

When the cut-off for parting of the patient population is taken at advanced fibrosis (F ≥ 3) or cirrhosis (F = 4), significant differences with lower fibrosis stages can still be seen, respectively p = 0.0079 and p = 0.0273 (Fig. 3a). However, the predictive character of sPDGFRβ strongly decreases (F ≥ 3: 0.6446 (95% CI: 0.5565–0.7327); F = 4: 0.6409 (95% CI: 0.5457–0.7360)) (Table 2) and is lower than the calculated established clinical scores (Table 2).

3.5. Predictive function of sPDGFRβ is independent of disease aetiology

Division of the patient cohort based on disease aetiology identified the strongest discriminative function of sPDGFRβ for significant liver fibrosis (F ≥ 2) in patients with alcoholic liver disease; 0.8634 (95% CI:
Fig. 2. PDGFRβ expression in a CCl4-induced mouse model of liver fibrosis. PDGFRβ-expression in total liver tissue of CCl4-injected mice, both on (a) WB and (b) immune histochemical staining. (a) PDGFRβ and αSMA protein levels were quantified and normalized versus GAPDH expression (n = 5) (whole western blot membrane for PDGFRβ is shown in Supplementary Fig. 1). Bars indicate the fold increase in CCl4-treated mice compared to healthy controls. (b) Representative images of PDGFRβ-staining on liver tissue of healthy and CCl4-induced mice are shown. Black bars represent 500 μm. The area of PDGFRβ positive staining was calculated by using image analysis software and is plotted as percentage of the total area (n = 5). (c) PDGFRβ mRNA expression in the non-parenchymal fraction (NPF) and different liver cell types, being quiescent HSCs, Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), and hepatocytes. Significant differences were calculated as compared to PDGFRβ mRNA expression in HSCs. Error bars represent mean values ± SD. (d) PDGFRβ expression by activated HSCs shown by co-staining of PDGFRβ and the activation marker αSMA in liver tissue of healthy or CCl4-injured mice. Representative images are shown (n = 3). Grey bars represent 100 μm.
The PRTA-score can predict better significant fibrosis (0.7849 (95% CI: 0.6995–0.8702) and advanced fibrosis (0.7470 (95% CI: 0.6586–0.8355), higher (Table 3) than Fib-4, APRI, and ALT/AST (Table 2). However, the predictive value for cirrhosis (0.7995 (95% CI: 0.7122–0.8868) remains lower that the AUC obtained by using the Fib-4 score: 0.8344 (95% CI: 0.7623–0.9605). The PRTA-score is independent of sex, as no significant differences (p = 0.9185) are observed between male (9.293 [5.538; 15.50]) and female (9.110 [7.280; 11.84]) patients. Additionally, no correlation has been found between BMI of the patients and outcome of the PRTA-score (r = 0.1064; p = 0.2558) (data not shown).

3.7. The PRTA-score is highly predictive for significant fibrosis independent of liver disease aetiology

We next compared the diagnostic accuracy of sPDGFRβ alone, with the PRTA-score, and with the most important, and most used, clinical score: Fib-4 scoring (Fig. 4). The prediction of significant fibrosis increased from 0.7303 (95% CI: 0.6395–0.8211) using sPDGFRβ alone (Table 2) to 0.7849 (95% CI: 0.6995–0.8702) using the PRTA-score (Table 3). With this cohort, the AUC is significantly higher than the AUC provided by the Fib-4 score: 0.6635 (95% CI: 0.5690–0.7581) (Table 2). Additionally, for prediction of significant fibrosis, using a cut-off value of 7.804, the PRTA-score had good sensitivity and specificity, respectively 77.1% and 73.17% (Table 3). When the patient population is divided based on aetiology of liver disease, a comparable significant predictive function for significant liver fibrosis is seen (Supplementary Table 5) for viral liver disease: 0.7905 (95% CI: 0.6395–0.8211) using sPDGFRβ alone (Table 2) to 0.7849 (95% CI: 0.6995–0.8702) using the PRTA-score (Table 3).

3.8. Validation of the PRTA-score in an independent patient cohort

We next validated the PRTA-score in an independent patient cohort of 57 patients (Supplementary Table 6), with NAFLD, with histological assessment of fibrosis stage. Analysis of this cohort further validated the high predictive value of the PRTA-score (0.7284 (95% CI: 0.5868–0.8700)) as compared to those obtained by applying sPDGFRβ levels’ 0.6702 (95% CI: 0.5192–0.8215) the Fib-4 score (0.6537 (95% CI: 0.4907–0.8168)) or NAFLD fibrosis score (0.6056 (95% CI: 0.4373–0.7737)) (Fig. 5a). Its important diagnostic value was further shown with its significant change (p = 0.00059) between patients with significant fibrosis (F ≥ 2) and those with no or mild fibrosis (F0–1) (Fig. 5b). The use of liver biopsy as reference method, allowed us to group patients with early stage liver fibrosis (F ≥ 2) into 2 populations: stage F1 and stage F2, to truly verify the accuracy of sPDGFRβ and the PRTA-score to distinguish between the early stages of liver fibrosis. While sPDGFRβ levels were up-regulated in a non-significant manner, the PRTA-score was significantly (p = 0.0386) higher in the patient cohort with stage F2 liver fibrosis, compared to those with
positive predictive value; NPV: negative predictive value.

the total patient cohort.

FibroScan to determine the stage of liver fibrosis progression. Correlation parameters were calculated with Spearman's correlations test.

Table 3

Accuracy of sPDGFRβ and the clinical scores Fib-4, APRI, and AST/ALT-ratio for the detection of significant fibrosis (F ≥ 2), advanced fibrosis (F ≥ 3), and cirrhosis (F = 4), in the total patient cohort.

|                  | AUC       | 95% CI        | p-value | Optimal Cut-off | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Youden's index |
|------------------|-----------|---------------|---------|-----------------|----------------|----------------|---------|---------|----------------|
| sPDGFRβ (pg/mL) | All patients |
| F ≥ 2            | 0.7303    | 0.6395 to 0.8211 | <0.0001 | 6003            | 82.47          | 62.00          | 80.49   | 65.03   | 0.4447         |
| F ≥ 3            | 0.6446    | 0.5565 to 0.7327 | 0.000252 | 5861            | 82.35          | 40.51          | 54.06   | 72.97   | 0.2286         |
| F = 4            | 0.6409    | 0.5457 to 0.7360 | 0.008703 | 7497            | 75.00          | 48.60          | 35.08   | 83.99   | 0.2360         |

Fib-4

All patients |
| F ≥ 2            | 0.6635    | 0.5690 to 0.7581 | 0.002295 | 1.495           | 65.52          | 68.18          | 79.66   | 50.97   | 0.3370         |
| F ≥ 3            | 0.7123    | 0.6225 to 0.8022 | <0.0001  | 1.495           | 74.58          | 62.5           | 62.83   | 74.31   | 0.3708         |
| F = 4            | 0.8344    | 0.7623 to 0.9065 | <0.0001  | 1.495           | 93.94          | 59.18          | 46.01   | 96.34   | 0.5312         |

APRI

All patients |
| F ≥ 2            | 0.6309    | 0.5331 to 0.7286 | 0.01466  | 0.4849          | 44.83          | 79.55          | 80.66   | 43.12   | 0.2438         |
| F ≥ 3            | 0.6723    | 0.5779 to 0.7667 | 0.0007139| 0.5718          | 44.07          | 87.5           | 74.98   | 64.79   | 0.3157         |
| F = 4            | 0.7965    | 0.7028 to 0.8902 | <0.0001  | 0.7002          | 60.61          | 90.82          | 70.98   | 86.16   | 0.5143         |

AST/ALT ratio

All patients |
| F ≥ 2            | 0.5976    | 0.4952 to 0.7001 | 0.07019  | 1.174           | 28.41          | 90.7           | 85.32   | 39.98   | 0.1911         |
| F ≥ 3            | 0.6423    | 0.5462 to 0.7383 | 0.005081 | 1.174           | 39.34          | 92.86          | 82.40   | 64.29   | 0.3220         |
| F = 4            | 0.7486    | 0.6458 to 0.8515 | <0.0001  | 1.184           | 55.88          | 91.75          | 71.49   | 84.88   | 0.4763         |

Table 4

Accuracy of PRTA-score, sPDGFRβ/albumin, sPDGFRβ/thrombocyte numbers, and sPDGFRβ for the detection of significant fibrosis (F ≥ 2), advanced fibrosis (F ≥ 3), and cirrhosis (F = 4), in the total patient cohort.

|                  | AUC       | 95% CI        | p-value | Optimal Cut-off | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Youden's index |
|------------------|-----------|---------------|---------|-----------------|----------------|----------------|---------|---------|----------------|
| PRTA-score       | All patients |
| F ≥ 2            | 0.7849    | 0.6995 to 0.8702 | <0.0001 | 7.804           | 77.11          | 73.17          | 84.54   | 62.69   | 0.5028         |
| F ≥ 3            | 0.7470    | 0.6586 to 0.8355 | <0.0001 | 9.979           | 70.91          | 78.26          | 73.49   | 75.99   | 0.4917         |
| F = 4            | 0.7995    | 0.7122 to 0.8868 | <0.0001 | 11.01           | 80.65          | 74.19          | 53.64   | 91.19   | 0.5484         |
| [sPDGFRβ/albumin (g/L)] | All patients |
| F ≥ 2            | 0.7431    | 0.6494 to 0.8369 | <0.0001 | 140.0           | 85.88          | 54.76          | 78.31   | 67.09   | 0.4064         |
| F ≥ 3            | 0.6702    | 0.5771 to 0.7633 | 0.001094 | 251.4           | 48.12          | 77.14          | 64.61   | 64.08   | 0.2626         |
| F = 4            | 0.6938    | 0.5902 to 0.7973 | 0.001097 | 253.4           | 59.18          | 73.68          | 45.52   | 83.04   | 0.3306         |
| [sPDGFRβ/(thrombocytes (/mm3)/100)] | All patients |
| F ≥ 2            | 0.7672    | 0.6808 to 0.8535 | <0.0001 | 3.707           | 69.32          | 77.27          | 85.29   | 56.97   | 0.4659         |
| F ≥ 3            | 0.7360    | 0.6487 to 0.8233 | <0.0001 | 4.039           | 75.00          | 70.83          | 68.61   | 76.92   | 0.4583         |
| F = 4            | 0.7701    | 0.6780 to 0.8622 | <0.0001 | 4.589           | 76.47          | 74.49          | 52.61   | 89.52   | 0.5096         |
| sPDGFRβ (pg/mL)  | All patients |
| F ≥ 2            | 0.7303    | 0.6395 to 0.8211 | <0.0001 | 6003            | 82.47          | 62.00          | 80.49   | 65.03   | 0.4447         |
| F ≥ 3            | 0.6446    | 0.5565 to 0.7327 | 0.000252 | 5861            | 82.35          | 40.51          | 54.06   | 72.97   | 0.2286         |
| F = 4            | 0.6409    | 0.5457 to 0.7360 | 0.008703 | 7497            | 75.00          | 48.60          | 35.08   | 83.99   | 0.2360         |

AUC: Area under the curve; CI: Confidence interval; PPV: positive predictive value; NPV: negative predictive value.

stage F1 liver fibrosis (Fig. 5c). Noteworthy, considering the high BMI values of these NAFLD-patients, we did not find any correlation between PRTA-levels and BMI-scores (r = 0.1282; p = 0.3601) (data not shown).

4. Discussion

Fibrosis progression is accompanied by increased PDGFRβ expression in activated HSCs [3]. We confirmed that PDGFRβ-expression is significantly dysregulated in human (Fig. 1d) and murine (Fig. 2a,b) subjects with liver fibrosis, and that HSCs are truly its most important source in the affected liver (Fig. 2c,d). We further investigated its diagnostic ability to detect significant fibrosis (F ≥ 2), advanced fibrosis (F ≥ 3), and cirrhosis (F = 4), in a patient population with different causes of liver disease. Circulating sPDGFRβ-levels were found to be highly elevated in patients with significant liver fibrosis (Fig. 3a), independently of the etiology of the liver disease. We increased its diagnostic accuracy by combining it with albumin levels and thrombocyte
numbers into a novel diagnostic algorithm: the PRTA-score. Next, the diagnostic value of sPDGFRβ levels and the PRTA-score were confirmed in an independent NAFLD-patient cohort, with histological staging of fibrosis in liver biopsies (Fig. 5).

Serological markers are considered the ideal way for diagnosis, thanks to their non-invasive quality, easy implementation in routine analysis, low cost, dynamic character, and no inter-and intra-observer variability [28]. We previously identified an enhanced presence of PDGFRβ on EVs extracted from the circulation of patients with early fibrosis (F ≤ 2) caused by chronic HBV or HCV infection [18]. Concentrating solely on EVs as biomarkers for fibrosis would significantly weaken the clinical relevance as the current clinical setting is not yet ready for implementation of EV analysis in its work flow [8]. We therefore focused on total circulating sPDGFRβ, either EV-bound or freely circulating, and showed that the sPDGFRβ levels have a very good diagnostic value for significant liver fibrosis when integrated into the PRTA-score. Since albumin and thrombocyte levels are already integrated in the clinical setting as easy, cheap, and commercially available biochemical analysis panels, we expect only limited additional costs for integration of the PRTA-score. As our scoring system solely relies on the analysis of circulating factors, it can be executed in almost every clinical centre, which is in strong contrast to the exclusive presence of liver biopsy and transient elastography in large and well-equipped hospitals. Additionally, compared to these latter diagnostic manners, our PRTA-score only requires limited human resources, which reduces the eventual costs on the patient and health care system. We thus anticipate that this easy and cost-effective PRTA analysis could facilitate screening of at-risk patients, and thus identify those patients who need liver biopsy for more specific evaluation of the extent and potential cause of liver injury. Eventually, this could lead to an earlier therapeutic intervention, leading to a decline in liver-associated morbidity and mortality and a further reduction of the current financial weight on the health care system.

One of the major weaknesses of current clinical and experimental scoring systems for liver fibrosis, is their limited or non-existing validation in multiple aetiologies of liver disease. More precisely, most of the diagnostic studies focus on patients with chronic viral hepatitis, and thus can only speculate its utility in patients with other causes of liver disease [29]. We therefore put focus on the aetiology-independence of our newly developed PRTA-score, as demonstrated by its high AUC values for the diagnosis of significant liver fibrosis in each aetiology-specific patient cohort (Supplementary Tables 3 and 5) superior to those obtained by the clinical scores Fib-4, APRI, and AST/ALT ratio (Supplementary Table 4). Additionally, the cut-off values of the PRTA-score for diagnosis of significant liver fibrosis (Supplementary Table 5) are comparable between viral liver disease (7.748), NAFLD (7.321), and alcoholic liver disease (7.738), in contrast to the high variability between aetiologies observed in the other clinical scores (Supplementary Table 4). Such aetiology-independent cut-off values, and the possibility to use one consensus standard cut-off value would create uniformity in all clinical centres, which is currently not the case for some diagnostic tools for liver fibrosis e.g. transient elastography [20]. Finally, we validated the aetiology-independence of our PRTA-score in an independent patient cohort, consisting of NAFLD-patients with extremely high BMIs (44–57 kg/m²). This cohort shows that potential negative effects of aetiology-specific characteristics, such as the high BMI and extensive liver steatosis did not affect the PRTA-score (Fig. 5). This study does have some limitations. In particular, the number of included patients is relatively low. However, despite these low patient numbers, there is a good correlation of the results over all 3 aetiologies of liver disease which affirms the obtained results and encourages further study of the diagnostic utility of the PRTA-score. An inequality in patient numbers representing each stage of the fibrosis progression is also present (Supplementary Table 2). In the initial population, especially the stages significant (n = 29) and advanced (n = 28) fibrosis were less represented, as compared to mild fibrosis (n = 51) and cirrhosis (n = 40), what could have led to some inconsistent cut-off values.

As our validation patient cohort solely included biopsy-staged NAFLD-patients, future prospective studies should focus on the further validation of the obtained results in biopsy-staged patients with other aetiologies of liver disease. Effectuating elastography measurements and serological assessment on patients which underwent liver biopsy, requires a substantial investment, but could provide information on the possibility to replace costly imaging modalities by the PRTA score. Additionally, it would be interesting to include patients from longitudinal studies, in which the cause of injury was eliminated. The resolution of liver fibrosis is based on a decline of activated HSCs in the liver, prior to degradation of extracellular matrix (ECM) [30]. Current non-invasive diagnostic tools fail in the sensitive and specific follow-up of liver resolution, which is not that surprising, considering they often rely on ECM content evaluation, as applies to the ELF score and most of the imaging
Acknowledgments

We would like to acknowledge Aneta Kozyra, Danielle Blyweert, and Iona De Mol for technical support. We thank Prof. Karin Vanderkerken for the use of the ZetaView® PMX110. We thank Dr. Bert Van den Bossche (Department of Hepatobiliary and Pancreas Surgery, ASZ Aalst) and prof. Daniel Jacobs-Tulleneers-Thevissen (Department of Thoracic and Transplantation Surgery and Surgical Oncology, University Hospital Brussels) for providing human tissue. We thank Prof. Niedergethmann and Dr. Hasenberg (Department for General- and Visceral Surgery, Alfried Krupp Hospital, Essen, Germany) for sample collection of the validation cohort. We also would like to thank Prof. Margarete Odenthal (Institute for Pathology, University Hospital Cologne), Prof. Hideo A. Baba (Institute for Pathology, University Hospital Essen), and Prof. Johannes Haybäck (Institute for Pathology, University Hospital Magdeburg) for histological preparation and assessments for the validation cohort.

Financial support

This work was supported by the Vrije Universiteit Brussel, the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Flanders) (HILIM-3D: SBO140045), and the Fund of Scientific Research Flanders (FWO) to LvG and IM. Funding sources had no involvement in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflicts of interest

JL and LvG are inventors of the pending patent “Soluble PDGFR beta as a biomarker for liver fibrosis”. No other potential conflicts of interest to disclose.

Author contributions

JL study concept and design; acquisition of data; analysis and interpretation of data; statistical analysis; drafting of the manuscript. SV analysis and interpretation of data. IM interpretation of data; critical revision of the manuscript. JPS, JB, and AC provision of samples; critical revision of the manuscript. HR provision of samples; interpretation of data; critical revision of the manuscript. LvG study concept and design; interpretation of data; critical revision of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.04.036.

References

[1] Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115(2):209–18.
[2] Pinzani M, Marra F. Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis 2001;21(3):397–416.

[3] Kobayashi P, Lade A, Lee YA, Dragomir AG, Sun Y, Fiel MI, et al. beta-PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis. J Hepatol 2015;63(1):141–7.

[4] Pinzani M. PDGF and signal transduction in hepatic stellate cells. Front Biosci 2002;7:1720–6.

[5] Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol 2002;97(10):2614–8.

[6] Cadzow JF, Rudy P. Receptors and signaling in hepatic stellate cells: results of a prospective nationwide survey. For the group of epidemiology of the French association for the study of the liver (AFEF). Hepatology 2000;32(3):477–81.

[7] Pasha T, Gabriel S, Therneau T, Dickson ER, Lindor KD. Cost-effectiveness of ultrasound-guided liver biopsy. Hepatology 1998;27(5):1220–6.

[8] Lambrecht J, Jan Poortmans P, Verhulst S, Reynaert H, Mannaerts I, van Grunsven LA. Prospects in performance of transient elastography for the staging of liver fibrosis: a systematic review and meta-analysis. Hepatology 2017;65(5):1557–65.

[9] Kennedy P, Wagner M, Castera L, Hong CW, Johnson CL, Sirlin CB, et al. Quantitative elastography methods in liver disease: current evidence and future directions. Radiology 2018;286(3):738–63.

[10] Sandrin L, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. Ultrasound Med Biol 2003;29(12):1705–13.

[11] Friedrich-Rust M, Nierhoff J, Lupsor M, Sporea I, Fierbinteanu-Braticevici C, Strobel D, et al. Performance of acoustic radiation force impulse imaging for the staging of liver fibrosis: a pooled meta-analysis. J Viral Hepat 2012;19(2):e212–9.

[12] Starkel P, Leclercq IA. Animal models for the study of hepatic fibrogenesis. Am J Pathol 1996;148(3):785–92.

[13] Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology 2006;43(6):1317–25.

[14] Williams AL, Hoodnagel JH. Ratio of serum aspartate to alanineaminotransferase in chronic hepatitis. Relationship to cirrhosis. Gastroenterology 1988;95(3):734–9.

[15] Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology 2003;38(2):518–26.

[16] European Association for Study of the Liver, Asociacion Latinoamericana para el Estudio del H. EASL–ALAT clinical practice guidelines: non-invasive tests for evaluation of liver disease severity and prognosis. J Hepatol 2015;63(1):237–64.

[17] Dalal PS, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, et al. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: systematic review and meta-analysis. Hepatology 2017;65(5):1557–65.

[18] Lambrecht J, Jan Poortmans P, Verhulst S, Reynaert H, Mannaerts I, van Grunsven LA. Circulating ECV-associated miRNAs as potential clinical biomarkers in early stage HBV and HCV induced liver fibrosis. Front Pharmacol 2017;8:56.

[19] Schwab R, Verhulst S, Roosens T, Oie C, Moya IM, Helder G, et al. Functionality based method for simultaneous isolation of rodent hepatic sinusoidal cells. Biomaterials 2017;139:91–101.

[20] Baranova A, Lal P, Birerdinc A, Younossi ZM. Non-invasive markers for hepatic fibrosis: liquid biopsy as the future gold standard? J Viral Hepat 2012;19(2):e212–9.

[21] Tacke F, Trautwein C. Mechanisms of liver injury, repair, and fibrosis: a cohort study. Gastroenterology 2011;140(2):319–31.

[22] Desmet VJ. Classification of chronic hepatitis: diagnosis, grading and staging. J Hepatol 1994;19(6):1513–20.

[23] Youden WJ. Index for rating diagnostic tests. Cancer 1950;3(1):32–5.

[24] Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200(4):373–783.

[25] Pinzani M, Marra F, Herbst H, DeFranco R, Grappone C, Gentilini A, et al. Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. Ann J Pathol 1986;148(3):785–800.

[26] Desmet VJ, Forns X, Mautone M, Chung RT, Fuchs BC. Noninvasive biomarkers of liver fibrosis: clinical applications and future directions. Curr Pathobiol Rep 2014;2(4):245–56.

[27] Tacke F, Trautwein C. Mechanisms of liver fibrosis resolution. J Hepatol 2015;63(4):1038–9.