A serologically assessed neo-epitope biomarker of cellular fibronectin degradation is related to pulmonary fibrosis

Hummersgaard Hansen, Annika; Wallem Breisnes, Helene; Skovhus Prior, Thomas; Hilberg, Ole; Guldager Kring Rasmussen, Daniel; Genovese, Federica; Vestergaard Lukassen, Marie; Svensson, Birte; Løcke Langholm, Lasse; Manon-Jensen, Tina

Total number of authors: 14

Published in:
Clinical Biochemistry

Link to article, DOI:
10.1016/j.clinbiochem.2023.110599

Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
A serologically assessed neo-epitope biomarker of cellular fibronectin degradation is related to pulmonary fibrosis

Annika Hummersgaard Hansen\textsuperscript{a,b,*,1}, Helene Wallem Breisnes\textsuperscript{a,b,1}, Thomas Skovhus Prior\textsuperscript{c,d}, Ole Hilberg\textsuperscript{e}, Daniel Guldager Kring Rasmussen\textsuperscript{a}, Federica Genovese\textsuperscript{a}, Marie Vestergaard Lukassen\textsuperscript{a}, Birte Svensson\textsuperscript{1}, Lasse Lecke Langholm\textsuperscript{a}, Tina Manon-Jensen\textsuperscript{a}, Morten Asser Karsdal\textsuperscript{a,1}, Diana Julie Leeming\textsuperscript{a}, Elisabeth Bendstrup\textsuperscript{c,d}, Marie Vestergaard Lukassen\textsuperscript{a}, Birte Svensson\textsuperscript{1}, Lasse Lecke Langholm\textsuperscript{a}, Tina Manon-Jensen\textsuperscript{a}, Morten Asser Karsdal\textsuperscript{a,1}, Diana Julie Leeming\textsuperscript{a}, Elisabeth Bendstrup\textsuperscript{c,d}, Jannie Marie Bülow Sand\textsuperscript{a}

\textsuperscript{a} Hepatic and Pulmonary Research, Nordic Bioscience, Herlev, Denmark
\textsuperscript{b} Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark
\textsuperscript{c} Center for Rare Lung Diseases, Department of Respiratory Diseases and Allergy, Aarhus University Hospital, Aarhus, Denmark
\textsuperscript{d} Department of Clinical Medicine, Aarhus University, Aarhus Denmark
\textsuperscript{e} Medical Department Vejle Hospital, Southern Danish University Hospital, Vejle, Denmark
\textsuperscript{f} Department of Biotechnology and Biomedicine, Technical University of Denmark, Denmark

ARTICLE INFO

Keywords:
Fibronectin
EDB
Neo-epitope
Extracellular matrix
Idiopathic pulmonary fibrosis
Biomarker
Serological biomarker
Non-invasive test

ABSTRACT

Background: Idiopathic pulmonary fibrosis (IPF) is characterized by excessive extracellular matrix (ECM) remodeling, herein ECM degradation. Fibronectin (FN) is an important component of the ECM that is produced by multiple cell types, including fibroblasts. Extra domain B (EDB) is specific for a cellular FN isoform which is found in the ECM. We sought to develop a non-invasive test to investigate whether matrix metalloproteinase 8 (MMP-8) degradation of EDB in cellular FN results in a specific protein fragment that can be assessed serologically and if levels relate to pulmonary fibrosis.

Method: Cellular FN was cleaved in vitro by MMP-8 and a protein fragment was identified by mass spectrometry. A monoclonal antibody (mAb) was generated, targeting a neo-epitope originating from EDB in cellular FN. Utilizing this mAb, a neo-epitope specific enzyme-linked immunosorbent assay (FN-EDB) was developed and technically validated. Serum FN-EDB was assessed in an IPF cohort (n = 98), registered at clinicaltrials.gov (NCT02818712), and in healthy controls (n = 35).

Results: The FN-EDB assay had high specificity for the MMP-8 degraded neo-epitope and was technically robust. FN-EDB serum levels were not influenced by age, sex, ethnicity, or BMI. Moreover, FN-EDB serum levels were significantly higher in IPF patients (median 31.38 [IQR 25.79–46.84] ng/mL) as compared to healthy controls (median 28.05 [IQR 21.58–33.88] ng/mL, p = 0.023).

Conclusion: We developed the neo-epitope specific FN-EDB assay, a competitive ELISA, as a tool for serological assessment of MMP-8 mediated degradation of EDB in cellular FN. This study indicates that degradation of EDB in cellular FN is elevated in IPF and warrants further investigation.

Abbreviations: AGC, Automatic gain control; BMI, body mass index; CV\%, coefficient of variance; DLCO\%, diffusing capacity of carbon monoxide in percentage of predicted value; ECM, extracellular matrix; EDA, extra domain A; EDB, extra domain B; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; FVC\%, forced vital capacity in percentage of predicted value; HRP, horseradish peroxidase; IPF, idiopathic pulmonary fibrosis; IQR, interquartile range; LLOD, lower limit of detection; LLOQ, lower limit of quantification; MS, mass spectrometry; MMPs, matrix metalloproteinases; mAb, monoclonal antibody; MRD, minimum required dilution; SD, standard deviation; MS\textsuperscript{2}, tandem mass spectrometry; FN I, type I repeats; FN II, type II repeats; FN III, type III repeats; ULOQ, upper limit of quantification; 6MWD, 6 min walk distance.

\* Corresponding author at: Herlev Hovedgade 205-207, 2730 Herlev, Denmark.
E-mail address: abh@nordicbio.com (A.H. Hansen).

1 Contributed equally.

https://doi.org/10.1016/j.clinbiochem.2023.110599

Received 23 September 2022; Received in revised form 19 April 2023; Accepted 14 June 2023

Available online 19 June 2023

0009-9120/© 2023 The Authors. Published by Elsevier Inc. on behalf of The Canadian Society of Clinical Chemists. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a complex chronic disease of unknown cause with a high mortality rate and significant impact on patients’ quality of life [1–3]. The rate of disease progression is hard to predict due to a high degree of heterogeneity between patients, and the overall median survival from time of diagnosis is below five years if untreated [4,5]. A hallmark of IPF is the dysregulation of the extracellular matrix (ECM) remodeling, resulting in excessive deposition of ECM proteins, including collagens and fibronectin (FN) [6,7]. Simultaneously, the expression of matrix metalloproteinases (MMPs) is upregulated [8,9], which results in a higher degree of ECM degradation compared to homeostatic conditions.

FN is a dimeric, extracellular glycoprotein with a size ranging from 233 to 270 kDa per monomer, depending on the isoform and post-translational modifications [10]. Generally, each monomer in FN consists of repetitive modular structures of 12 type I repeats (FN I), two type II repeats (FN II), and 15–17 type III repeats (FN III), where the flexible FN III allows for either a stretched or compact protein conformation (Fig. 1) [11,12]. FN is an ubiquitous ECM protein that is involved in a diverse range of critical biological processes, such as ECM assembly, cell adhesion, cell signaling, migration, wound healing, and cell differentiation [11–15]. Different isoforms and domains of FN enables it to engage with other FN molecules, other ECM proteins, and integrins [16]. Soluble and insoluble forms exist and are referred to as plasma and cellular FN, respectively. Cellular FN is produced locally by different cell types, such as fibroblasts, and incorporated into the ECM or produced as a response to tissue damage [17–20]. Depending on splicing, cellular FN may contain certain FN III domains, namely extra domain A (EDA) and extra domain B (EDB). The biological function of EDA and EDB has not yet been fully elucidated [21,25], however, EDB has shown to significantly influence phagocytosis [22], osteoblast differentiation [21], fibroblast growth, and ECM assembly [23–25]. Moreover, EDB has been seen contributing to wound healing [26], angiogenesis, and endothelial cell proliferation [27].

In IPF, the most studied MMP family member is MMP-7 due to its potential as a blood biomarker [28,29]. However, MMP-8 has also shown to be significantly increased in IPF patients as compared to controls when assessed serologically [30]. Studies have indicated MMP-8 as a profibrotic mediator when investigated in MMP-8-null mice with bleomycin-induced pulmonary fibrosis [31]. In vitro studies have suggested EDB to be especially susceptible to proteolytic degradation and could also be a susceptible target for MMPs [32].

In this study, we aimed to investigate if MMP-8 mediated degradation of EDB in cellular FN was increased in IPF patients compared to healthy controls. The objectives were to 1) identify a target-site within EDB by in vitro cleavage with MMP-8, 2) develop a neo-epitope-specific assay for detection of the identified fragment, and 3) evaluate biological relevance in serum samples from IPF patients and compare levels to healthy controls.

2. Material and methods

2.1. In vitro cleavage of cellular fibronectin by matrix metalloproteinase 8

Cellular FN fragments were generated in vitro by mixing activated...
native MMP-8 from stimulated human neutrophils (cat. no. 444229, Merck, Whitehouse Station, NJ, USA) with cellular FN protein from human foreskin fibroblasts (cat. no. F2518, Sigma Aldrich, St. Louis, MO, USA). The cellular FN concentration was 0.33 mg/mL in a final volume of 75 µL, where protein/protease molar ratio was 100:1. The mix was incubated for 24 h at 37 °C, and the reaction was stopped by addition of 2 µL 5 mM ethylenediaminetetraacetic acid and stored at −20 °C until analysis. Samples were analyzed in triplicates, where samples containing MMP buffer, MMP-8 without cellular FN, and cellular FN without MMP-8 were prepared as experimental controls.

2.2. Cellular fibronectin extra domain B neo-epitope identification

In vitro cleavage samples were desalted by stage-tipping before tandem mass spectrometry (MS²) analysis. For the liquid chromatography MS² procedure, each sample was loaded onto a 2 cm C18 trap column (Thermo Fisher 164705), connected in-line to a 15 cm C18 reverse-phase analytical column (Thermo EasySpray E8903) using 100% Buffer A (0.1% formic acid in water) at 750 bar, using the Thermo EasyLC 1000 HPLC system, and the column oven operating at 45 °C. Peptides were eluted over a 120 min gradient ranging from 6 to 60% of 80% acetonitrile, 0.1% formic acid at 250 nL/min, and the Q-Exactive instrument (Thermo Fisher Scientific) was run in a DD-MS² top10 method. Full mass spectrometry (MS) spectra were collected at a resolution of 70,000, with an automatic gain control (AGC) target of 5 × 10⁶ or maximum injection time of 20 ms and a scan range of 300–1750 m/z. The MS² spectra were obtained at a resolution of 17,500 with an AGC target value of 1 × 10⁶ or maximum injection time of 60 ms, a normalized collision energy of 25 and an intensity threshold of 1e⁶. Dynamic exclusion was set to 30 s, and ions with a charge state <2 or unknown were excluded. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility.

Raw files from liquid chromatography MS² analyses were analyzed using the MaxQuant (version 1.5.1.2, München, Germany) search engine, with the following search criteria: enzyme mode: unspecific; maximum missed cleavages: 2; modifications: acetyl (protein N-term), oxidation (M), carbamidomethyl (C); minimum peptide length: 7 amino acids. The search was run against the human FN sequence from UniProt (accession number P02751, downloaded 22-05-2015). The identified peptides were filtered by MS² fragmentation and aligned using Basic Alignment Search Tool (BLAST).

2.3. Monoclonal antibody production and clone characterization

Six- to seven-week-old female Balb/C mice (Harlan/Envigo, Venray, Netherlands) were immunized subcutaneously with 100 µg immunogenic peptide conjugated to Keyhole limpet hemocyanin (KLH) (TGLEGPGIDYD-GGC-KLH) emulsified in 100 µL Gerbu MM Adjuvant (cat. no. 3001, Gerbu Biotechnik, Heidelberg, Germany). The immunizations were repeated with three-week intervals until stable serum titer levels were reached. The mice with the highest and best reactivity serum titer were selected for intraperitoneal boosting. Three days prior to isolation of the spleen, the mice were boosted intraperitoneally with 100 µg immunogenic peptide dissolved in ultrapure water. Hybridoma cells were produced by fusing spleen cells with SP2/0 myeloma cells as described previously [33] and cloned in culture dishes using hypoxanthine-aminopterin-thymidine selection medium. The hybridoma clones were cultured in 96-well microtiter plates and the standard limited dilution method was used to ensure monoclonal growth. Supernatant was collected from the antibody-producing hybridoma cells and screened for reactivity using a biotinylated peptide (TGLEGPGIDYD-K-Biotin), and a standard peptide (TGLEGPGIDYD) was used as a calibrator to test the specificity of the monoclonal antibody (mAb). The mAb with the best reactivity and specificity towards the standard peptide was selected for assay development and purified using a HiTrap Affinity Protein G column (cat. no. GE17-0404-01, Merck). The purified mAb was labelled with horse-radish peroxidase (HRP) using a peroxidase labelling kit (cat. no. 11829696001, Roche, Basel, Switzerland) according to the manufacturer’s instructions. To evaluate the specificity of the selected mAb, the binding to standard-, elongated- (VTGLEPGIDYD), truncated- (GLEPGIDYD), see Table 1, and other unrelated peptides (non-sense standard- and non-sense coating-peptide) targeting a different domain in FN were tested. All peptides were purchased from Genscript (Piscataway, NJ, USA) with a ≥ 95% purity.

2.4. FN-EDB competitive enzyme-linked immunosorbent assay procedure

For the enzyme-linked immunosorbent assay (ELISA) procedure, a 96-well streptavidin-coated microwell plate (cat. no. 655995, Greiner Bio-One, Kremsmünster, Austria) was coated with 100 µL biotinylated peptide dissolved in coating buffer (25 mM phosphate-buffered saline (PBS) with bovine serum albumin, tween-20, and bromidox (BTB), 8 g/L NaCl, pH 7.4) and incubated for 30 min at 20 °C with shaking at 300 rpm. A 10-points standard curve was prepared with a 2-fold dilution of 300 ng/mL standard peptide and human serum samples were prediluted 1:2 in incubation buffer (25 mM PBS-BTB, 8 g/L NaCl, 5% Liquid II (cat. no. 11941836001, Roche), pH 7.4). The plate was washed 5 times with washing buffer (25 mM Tris, 50 mM NaCl, pH 7.2) before the addition of 20 µL of standard peptide, assay controls, and samples in duplicates to appropriate wells, followed by 100 µL HRP-conjugated mAb in incubation buffer. The plate was incubated for 20 h at 4 °C with shaking at 300 rpm and thereafter washed 5 times with washing buffer. Finally, 100 µL chemiluminescence substrate (cat. no. 11582950001, Roche) was added to the wells and incubated in darkness for 3 min before reading. Relative light units emitted from the plate was read using SpectraMax i3x (Molecular Devices, San Jose, CA, USA) and the standard curve was plotted using a four-parametric logistic fit model.

2.5. Technical evaluation of FN-EDB

The precision of the FN-EDB assay was assessed by determining the intra- and inter-assay variations. Ten human serum samples were measured in double determination in ten independent runs, and the mean coefficient of variance (CV%) within the plate was used to find the intra-assay variation, while the mean CV% between the plates was used to calculate the inter-assay variation. Acceptable intra- and inter-assay variations were ≤10% and ≤15%, respectively. Linearity was determined by calculating the recovery percentage of four human serum samples in 2-fold serial dilutions when compared to the minimum required dilution (MRD). Interference was investigated by spiking three human serum samples with 2.5 and 5.0 mg/mL hemoglobin (cat. no. H7379, Sigma Aldrich), 1.5 and 5.0 mg/mL intralipids (cat. no. I141, Sigma Aldrich), or 5.0, 10, 20, 40, 60, 80, and 100 ng/mL biotin (cat. no. B4501, Sigma Aldrich) and compared to a non-spiked reference. To determine the stability of the analyte, three human serum samples were subjected to five freeze/thaw cycles with the first cycle as reference or

<table>
<thead>
<tr>
<th>FN-EDB</th>
<th>Antigen</th>
<th>TGLEGPGIDYD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immune</td>
<td>TGLEGPGIDYD-GGC-KLH</td>
</tr>
<tr>
<td></td>
<td>Biotinylated peptide</td>
<td>TGLEGPGIDYD-K-Biotin</td>
</tr>
<tr>
<td></td>
<td>Standard peptide</td>
<td>TGLEGPGIDYD</td>
</tr>
<tr>
<td></td>
<td>Elongated peptide</td>
<td>TGLEGPGIDYD</td>
</tr>
<tr>
<td></td>
<td>Truncated peptide</td>
<td>TGLEGPGIDYD</td>
</tr>
</tbody>
</table>

Table 1

FN-EDB assay-related peptides. A 10 amino acid sequence in extra domain B of cellular fibronectin was identified (antigen) and used for development of monoclonal antibody and peptides needed to test antibody specificity. The synthetic peptides included in the project were immunogenic-, coating-, standard-, elongated-, and truncated peptide.
stored for up to 48 h at 4 °C and 20 °C and compared to a reference sample stored at −20 °C. The acceptable recovery percentage for accuracy, linearity, interference, and analyte stability tests was 100 ± 25%.

2.6. Patient description

Serum samples were collected from 35 healthy controls and 98 prevalent IPF patients after informed consent. Healthy control samples were purchased at Lee Biosolutions (cat. no. 991-24-PS, Maryland Heights, MO, USA). Samples were collected in 2018 and processed immediately after collection according to standard operating procedures and stored at −80 °C until analysis. IPF patients were recruited, and baseline serum samples collected from August 2016 to March 2018. The IPF cohort has previously been described [34,35] (cohort registered at clinicaltrials.gov [NCT02818712] and approved by the Central Denmark Region Committee on Health Research Ethics [case no. 1-10-72-87-16]). Inclusion criteria for IPF patients were ≥18 years of age and an IPF diagnosis based on international guidelines and multidisciplinary discussions [36,37]. Exclusion criteria were intellectual or linguistic barriers that prevented the completion of questionnaires.

2.7. Statistical analyses

All statistical analyses were performed using GraphPad Prism (version 9.1.2, San Diego, CA, USA). Data were examined for normality using D’Agostino-Pearson omnibus test. When comparing two groups, Mann-Whitney test was used and when comparing three or more groups, Kruskal-Wallis One-Way ANOVA with Dunn’s multiple comparisons test was used. For correlation test and chi-square test, correlation coefficient [r] = −0.156; p = 0.371, as shown in Fig. 3. In addition, ethnicity did not indicate to have an effect on the levels of FN-EDB in healthy controls (median in Black 24.21 [IQR 18.81–29.03] ng/mL, Caucasian 30.02 [IQR 25.99–40.59] ng/mL, and Hispanic 25.72 [IQR 22.55–30.95] ng/mL; p = 0.117). Associations to sex or age were also not seen in the IPF population, nor were there associations to anti-fibrotic treatment status (p = 0.929), forced vital capacity in percentage of predicted value (FVC%) (p = 0.946), diffusing capacity for carbon monoxide in percentage of predicted value (DLCO%) (p = 0.985), 6-minute walk distance (6MWD) (p = 0.281), or BMI (p = 0.621) (Supplementary Data Fig. S 1).

3. Results

3.1. Cellular fibronectin neo-epitope identification

Unique peptide fragments were identified with MS analysis of in vitro MMP-8 cleaved FN, where one fragment originated from EDB, TGLEGIDYDISVI1329-1339. The fragment was an MMP-8 generated neo-epitope and could not be detected by MS in control samples consisting either solely of the cleavage buffer, the MMP-8 solution, or full-length cellular FN. The summary MS data of the unique fragment within EDB can be seen in Table 2. The fragment’s amino acid sequence uniqueness was assessed by BLAST and showed no match to other proteins, and the sequence was 100% homologues between human, rat, mouse, and bovine (data not shown).

3.2. Technical validation of FN-EDB

The specificity of the FN-EDB competitive ELISA was tested and demonstrated reactivity only towards the standard peptide, and no reactivity towards the elongated-, truncated-, and non-sense standard-peptide (Fig. 2), indicating that the FN-EDB assay was neo-epitope specific toward the selected MMP-8 cleavage site. Lastly, the mAb had no reactivity towards the non-sense coating peptide and did not yield a signal.

The technical performance of the FN-EDB assay was evaluated by determining the intra- and inter-assay variations, linearity, analyte stability, and interference. A summary of the data is shown in Table 3. The measurement range of the FN-EDB ELISA was determined by lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), providing a range from 5.76 to 150 ng/mL in serum, with a lower limit of detection (LOD) of 5.24 ng/mL. The technical performance was precise, as shown by acceptable intra- and inter-assay variations with means of 8.4% and 10.6%, respectively, and the linearity of serum samples was accepted in 1:2 dilution from the MRD. The recovery percentage was accepted for standard peptide spiked in serum, high serum samples spiked in low serum samples, serum samples that underwent up to five freeze–thaw cycles, and serum samples that were stored at 4 °C and 20 °C for up to 48 h. Hemoglobin and intralipid interference were investigated in serum samples, where no interference could be observed at low or high concentrations. Interference from biotin was also investigated, where non was seen at low concentrations, however, high concentrations of above 60 ng/mL had an impact on serum sample measurements (data below 60 ng/mL not shown).

3.3. Basic demographics

The 35 healthy controls had a mean age of 55.0 (SD 2.9) years and 48.6% were male (Table 4). Moreover, 48.6% were Caucasian (n = 17), 25.7% were Black (n = 9), and 25.7% were Hispanic (n = 9). The mean age of the 98 IPF patients was 72.6 (SD 6.0) years with a body mass index (BMI) of 26.2 (SD 3.9). The population consisted of 80.6% males and 100% were Caucasian. The distribution of sex and age was significantly different between healthy controls and IPF patients (p < 0.0001). Additionally, 50 and 31 patients were on anti-fibrotic treatment with Pirfenidone or Nintedanib respectively, and 17 patients were untreated at the time of sample collection.

3.4. FN-EDB was elevated in IPF and not affected by basic demographics

Levels of serum FN-EDB in healthy controls were not affected by sex (median 25.83 [IQR 18.02–30.65] ng/mL for females vs. 29.64 [IQR 22.96–46.14] ng/mL for males; p = 0.088), or age (correlation coefficient [r] = −0.156; p = 0.371), as shown in Fig. 3. In addition, ethnicity did not indicate to have an effect on the levels of FN-EDB in healthy controls (median in Black 24.21 [IQR 18.81–29.03] ng/mL, Caucasian 30.02 [IQR 25.99–40.59] ng/mL, and Hispanic 25.72 [IQR 22.55–30.95] ng/mL; p = 0.117). Associations to sex or age were also not seen in the IPF population, nor were there associations to anti-fibrotic treatment status (p = 0.929), forced vital capacity in percentage of predicted value (FVC%) (p = 0.946), diffusing capacity for carbon monoxide in percentage of predicted value (DLCO%) (p = 0.985), 6-minute walk distance (6MWD) (p = 0.281), or BMI (p = 0.621) (Supplementary Data Fig. S 1).

When comparing healthy controls to IPF patients, serum FN-EDB levels were significantly elevated in patients with IPF with a median concentration of 31.38 [IQR 25.79–46.84] ng/mL as compared to healthy controls (median 28.05 [IQR 21.58–33.88] ng/mL, p = 0.023) (Fig. 4).

4. Discussion

In the current study, we developed and validated the competitive ELISA FN-EDB, which is specific for a neo-epitope generated by MMP-8 mediated degradation of EDB in cellular FN. The overall findings of this study were that 1) a technically robust ELISA was developed with high specificity towards the FN-EDB neo-epitope, 2) FN-EDB serum levels
were not affected by sex, age, BMI, or ethnicity, 3) FN-EDB serum levels were significantly increased in IPF patients when compared to healthy controls.

The FN-EDB ELISA was technically robust and showed good accuracy, precision, linearity, and stability. High specificity towards the FN-EDB neo-epitope was demonstrated by showing good reactivity to the standard peptide and no reactivity to the elongated or truncated peptides. Moreover, no reactivity was observed towards the non-sense peptides which targeted a different domain in FN, further strengthening the specificity of the mAb towards the neo-epitope.

The neo-epitope analyte was shown to be stable in serum after repeated freeze–thaw cycles and when stored at 4°C and 20°C for up to 48 h, indicating that the neo-epitope was not accumulated or degraded due to sample handling. Hemoglobin, lipids, and biotin are common native compounds that may result in assay interference, thus, we sought to investigate whether these compounds influenced FN-EDB [38,39]. Results indicated no interference from hemoglobin or lipids, however, biotin concentrations >60 ng/mL did interfere with FN-EDB measurements in serum. Biotin is known to potentially interfere with immunoassays employing biotinylated peptides, such as FN-EDB. However, the concentration of circulating biotin is typically within the range of 0.1–0.8 ng/mL [40], implying that the concentration that could interfere with FN-EDB measurements is seldom reached in clinical settings.

Previously, it has been established that different neo-epitopes originating from the same protein can have different implications and relevancies in pathologies [41–43]. Thus, the neo-epitope specific FN-EDB assay has the potential to elucidate whether MMP-8 mediated degradation of EDB in cellular FN is related to disease pathologies. To investigate this, FN-EDB was measured in a cohort of patients with IPF. We found significantly elevated FN-EDB levels in serum when compared to healthy controls, indicating that degradation of EDB by MMP-8 is a pathological event in IPF. The cohorts were non-matched; however, there seemed to be no effect of age, BMI, ethnicity, or sex on FN-EDB levels, indicating that the differences observed between IPF patients and healthy controls were related to pathology.

Patient characteristics of healthy controls and patients with idiopathic pulmonary fibrosis. IPF: idiopathic pulmonary fibrosis, SD: standard deviation, BMI: body mass index, FVC: forced vital capacity, DLCO: diffusing capacity for carbon monoxide, 6MWD: 6-minute walk distance.

Table 4

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Healthy</th>
<th>IPF</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>35</td>
<td>98</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age (years), mean ± SD</td>
<td>55.0 ± 2.9</td>
<td>72.6 ± 6.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>17 (48.6%)</td>
<td>79 (80.6%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± SD</td>
<td>–</td>
<td>26.2 ± 3.9</td>
<td>–</td>
</tr>
<tr>
<td>Ethnicity, n (Caucasian/Black/Hispanic)</td>
<td>(17/9/9)</td>
<td>(98/0/0)</td>
<td>–</td>
</tr>
<tr>
<td>Time since diagnosis (months), mean ± SD</td>
<td>–</td>
<td>21.07 ± 17</td>
<td>–</td>
</tr>
<tr>
<td>Treatment status, n (Pirfenidone/Nintedanib/None)</td>
<td>–</td>
<td>50/31/17</td>
<td>–</td>
</tr>
<tr>
<td>FVC (% predicted), mean ± SD</td>
<td>–</td>
<td>83.5 ± 24.2</td>
<td>–</td>
</tr>
<tr>
<td>DLCO (% predicted), mean ± SD</td>
<td>–</td>
<td>46.4 ± 14.4</td>
<td>–</td>
</tr>
<tr>
<td>6MWD (meters), mean ± SD</td>
<td>–</td>
<td>447.8 ± 125.2</td>
<td>–</td>
</tr>
</tbody>
</table>
isoforms, however, it has been shown to be elevated in certain cancers and within fibrotic tissue, and to be localized in a different manner than the EDA isoforms in tissue of patients with renal diseases [44, 45]. Additionally, cellular FN containing EDB can be embedded in the ECM or released from the ECM as part of a tissue repair response and aberrant inflammation [18–20]. Further studies are needed to determine whether the generation of this neo-epitope is dependent on processes occurring during tissue homeostasis, tissue repair, and/or associated with a wound healing response. Increased ECM production is a hallmark in IPF, wherein TGF-β1 is one of the central profibrotic cytokines [46]. TGF-β1-treated fibroblasts have shown to increase the production of both EDA and EDB isoforms [47–49]. Thus, a higher production of FN with EDB could lead to a higher amount being subject to degradation, which could partially explain why FN-EDB is elevated in IPF. Additionally, a more inflammatory environment with higher concentration of cells releasing MMP-8, such as in IPF lungs, could explain the higher systemic levels of FN-EDB. To investigate whether the increased systemic levels of FN-EDB is specific for the EDB isoform, it could be interesting for future studies to compare FN-EDB to a similar neo-epitope assay that targets an MMP-8 generated fragment of EDA.

In IPF, tissue rigidity may increase in part due to ECM protein modifications, such as cross-linking, induced by ECM modifying enzymes [50]. Due to the high number of tyrosine residues in the primary structure of cellular FN, the protein has been shown to be prone to di-tyrosine cross-linking, decreasing its susceptibility to proteolytic degradation [51]. The fragment identified in the present study contained a tyrosine residue that could limit the release of the neo-epitope if the fragment was cross-linked. Despite this consideration, a significant increase was still observed in IPF patients when compared to healthy controls.

The release of the FN-EDB neo-epitope in vitro was dependent on MMP-8; a protease that has been suggested to be a pro-fibrotic mediator and which has been found elevated in studies investigating the mRNA and protein expression in IPF [30, 31]. However, it cannot be excluded that other proteases might generate this neo-epitope. Thus, further studies looking into proteases and their ability to generate the FN-EDB neo-epitope would be highly interesting, particularly proteases that have previously been suggested to play a role in IPF, such as MMP-7 [28].

Limitations of this study included non-matched groups, however, FN-EDB levels were not influenced by sex or age, and similarly no confounding effects on FN-EDB were indicated by BMI or ethnicity in IPF patients or healthy controls, respectively. Moreover, the IPF cohort included prevalent patients at time of recruitment, and patients followed...
different treatment strategies, which may add an additional factor to a complex disease with many subtypes. Recently, it has been demonstrated that anti-fibrotic treatment may alter ECM remodeling in IPF [52]. This may have influenced FN-EDB levels in IPF patients receiving anti-fibrotic treatment as compared to incidental, treatment-naïve patients. Investigating FN-EDB in incidental IPF patients longitudinally may establish if biomarker levels are affected by therapeutic intervention and/or related to disease activity.

5. Conclusion

In this study, we developed and validated a neo-epitope assay, FN-EDB, specific for an MMP-8 mediated degradation fragment within EDB of cellular FN. Serum FN-EDB levels were shown to be increased in IPF patients compared to healthy controls, supporting that EDB is subject to a higher degree of degradation in IPF than in physiological conditions. Future studies are needed to validate these findings as well as investigate longitudinal levels of FN-EDB in incidental IPF patients.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. H. Hansen, D. G. K. Rasmussen, F. Genovese, L. L. Langholm, T. Manon-Jensen, D. J. Leeming, M. A. Karsdal, and J. M. B. Sand, are employed at Nordic Bioscience which is a company involved in discovery and development of biochemical markers. Moreover, D. G. K. Rasmussen, T. Manon-Jensen, M. A. Karsdal, D. J. Leeming, L. L. Langholm and J. M. B. Sand are shareholders in Nordic Bioscience Holding A/S. H.W. Breisnes and technical-staff involved in acquiring and supplying samples and measurements. Additionally, we wish to acknowledge Lene Holberg Blicher for their help with mass spectrometry measurements and analysis. Lastly, we wish to acknowledge all the organizations who funded this study.

Data availability

The data obtained in the current study are included in this published article, available upon request.

Funding

The Q-Exactive mass spectrometer was granted by Independent Research Fund Denmark | Natural Sciences (ID: DFF – 1335-00071 to BS). Moreover, this study has been funded by the Danish Research Foundation, TrygFonden (grant 118860), Aarhus University (unrestricted), Boehringer Ingelheim Denmark (unrestricted), the Danish Lung Association’s Fund, the Health Research Fund of the Central Denmark Region, and the Ellen and Knud Dalhoff Larsen’s Fund. The funders of the study had no role in study design, data collection, data analyses, data interpretation, writing of the report or decision to submit the paper for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2023.110599.

References
