





An MMP-degraded and cross-linked fragment of type III collagen as a non-invasive biomarker of hepatic fibrosis resolution

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Funding information

Research Foundation

Handling Editor: Luca Valenti

Abstract

Background and Aims: Liver fibrosis results from a prolonged wound healing response to continued injury with excessive production of extracellular proteins. In patients with chronic liver disease, the monitoring of liver fibrosis dynamics is of high interest. Whilst markers of fibrogenesis exist, markers of hepatic fibrosis resolution remain an unmet clinical need. Thus, we sought to develop an assay quantifying a circulating proteolytic fragment of cross-linked type III collagen as a biomarker of fibrolysis, testing its utility in two clinical cohorts of liver fibrosis of distinct aetiology and regressing endotype

Methods: We used a monoclonal antibody targeting the C-telopeptide of type III collagen following C-proteinase cleavage to develop and validate a neo-epitope-specific enzyme-linked immunosorbent assay (CTX-III). A potential fibrosis resolution marker, CTX-III, was measured in two clinical cohorts of patients with obesity-associated non-alcoholic fatty liver disease undergoing bariatric surgery or hepatitis C virus infection from a clinical trial study evaluating the anti-fibrotic effect of farglitazar.

Results: CTX-III was robust and specific for the targeted neo-epitope with good reproducibility in EDTA plasma. We assessed type III collagen remodelling using a panel of biomarkers, including a type III collagen formation marker (PRO-C3), degradation (C3M), and CTX-III (fibrolysis). Net fibrolysis was increased in patients with non-alcoholic fatty liver disease following bariatric surgery ($p < .001$). Moreover, net fibrolysis identified spontaneous fibrotic regressors from stable and progressors ($p < .05$ and $p < .001$) among hepatitis C virus infection patients.

Abbreviations: AST, aspartate aminotransferase; BMI, body mass index; CLD, chronic liver disease; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; HSCs, hepatic stellate cells; LLOD, lower limit of detection; MMP, matrix metalloproteinase; NAFLD, non-alcoholic fatty liver disease; SD, standard deviation; SIAJ, scar-in-a-jar; SVR, sustained virologic response; ULOD, upper limit of detection.

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Conclusion: Circulating CTX-III as a marker of fibrolysis indicates the biomarker's beneficial use in assessing hepatic fibrosis resolution.

KEYWORDS

collagen cross-linking, fibrosis resolution, hepatic fibrosis, non-invasive biomarkers

1 | BACKGROUND

Liver-associated deaths are increasing worldwide, and fibrosis is a significant prognostic factor impacting disease progression and liver transplantation.^{1,2} There are no specific treatments for fibrosis, partly because of the lack of non-invasive biomarkers to monitor therapeutic efficacy. Similarly, identifying patients at risk for liver fibrosis progression may be a clinically helpful tool.^{3,4} Elastography and imaging techniques have been used to screen advanced fibrosis, and liver biopsy remains the gold standard diagnostic tool but is limited by risk, cost, and complexity.¹⁻⁶

The leading causes of chronic liver disease and fibrosis are non-alcoholic fatty liver disease (NAFLD), affecting one-quarter of the world population,⁷⁻⁹ and hepatitis C virus (HCV) infection.¹⁰ HCV remains highly prevalent in countries with limited economic resources, poor diagnosis, and limited access to direct-acting antivirals, which could cure the infection and prevent transmission.^{11,12} Fighting metabolic diseases, obesity and diabetes, and infection are the more direct strategies to solve the issue. Understanding the pathogenesis of fibrosis progression could aid treatments and clinical assessment. It is essential to quantify tissue formation (fibrogenesis) and degradation (fibrolysis) to evaluate the treatment efficacy of anti-fibrotic drugs in clinical trials, as successful tissue regeneration will counter-balance both.

In chronic liver disease (CLD), there is an imbalanced response to injury,¹³ in which the activation of fibroblasts into myofibroblasts results in excessive collagen production, disruption of the extracellular matrix (ECM), and excessive inflammatory response,^{14,15} eventually resulting in liver fibrosis. Over time, the heavily cross-linked and non-compliant ECM perpetuates fibrosis.¹⁶ In the fibrotic ECM, (myo)fibroblast-derived type III collagen is proteolytically cleaved with subsequent enzymatic cross-linking within the collagen fibrils, which is a critical mechanism in fibrosis progression (Figure 1).¹⁷ Based on this knowledge, we have previously designed blood-based surrogate markers to explore fibrogenesis such as PRO-C3, a marker of fibroblast activity,¹⁸ and inflammation-associated type III collagen degradation by matrix metalloproteinase (MMP) (C3M).^{19,20} Following previously described strategies already successful in diseases characterised by excessive remodelling of cross-linked collagens,^{21,22} we envision that fibrogenic and cross-linked fibrolytic biomarkers may improve the diagnosis, prognosis, and evaluation of therapeutic procedures in chronic liver disease patients. Herein, we assessed the role of the novel CTX-III biomarker in assessing cross-linked type III collagen degradation, PRO-C3 and C3M in patients with obesity-induced NAFLD undergoing bariatric surgery and patients with HCV undergoing farglitazar therapy.

Lay Summary

Measuring blood-based fragments reflecting the breakdown (CTX-III) and build-up (PRO-C3) of type III collagen may provide a non-invasive tool for assessing the bettering or worsening of liver fibrosis. The use of these biomarkers could potentially negate invasive liver biopsies.

2 | METHODS

2.1 | Clinical cohorts

We have analysed two clinical cohorts of patients with different causes and degrees of liver fibrosis. Fibrosis was assessed in paired plasma samples in 68 consecutive patients with obesity-induced NAFLD programmed for bariatric surgery (Cohort 1). Blood was sampled immediately before surgery and again at six months follow-up. Clinical procedures have been described elsewhere.²³ All patients provided an intraoperative wedge-liver biopsy that was histologically assessed using the NAS score.²⁴

The second cohort included 158 patients with HCV-associated liver fibrosis (Cohort 2) initially recruited for the phase II, randomised, double-blinded, placebo-controlled multicenter study evaluating the effects of farglitazar (ClinicalTrials.gov identifier NCT00244751). The study of farglitazar, a PPAR- γ agonist, did not reach its endpoint.²⁵ At the initial screening, a liver biopsy was taken 120 days before treatment initiation. Following a 52-week period, a second biopsy was taken. Liver fibrosis was assessed according to the Ishak scoring system.²⁶ Only plasma samples obtained at the initial screening were used for biomarker analysis in this study, with 46 of the 158 patients being in the placebo group. The 46 patients of the placebo group were assessed for their spontaneous endotype by calculating the delta Ishak stage from the initial screening to the second follow-up biopsy: Regressive (Δ Ishak stage ≤ -1), Stable (Δ Ishak stage = 0), and Progressive (Δ Ishak stage ≥ 1).

A schematic overview of patient demographics for both cohorts is provided in Tables 2 and 3 respectively. EDTA plasma was stored at -80°C until analyses. All patients provided written consent, with studies performed following the ethical principles of the Declaration of Helsinki. Approval was obtained by the respective Institutional boards and their Ethics committees.

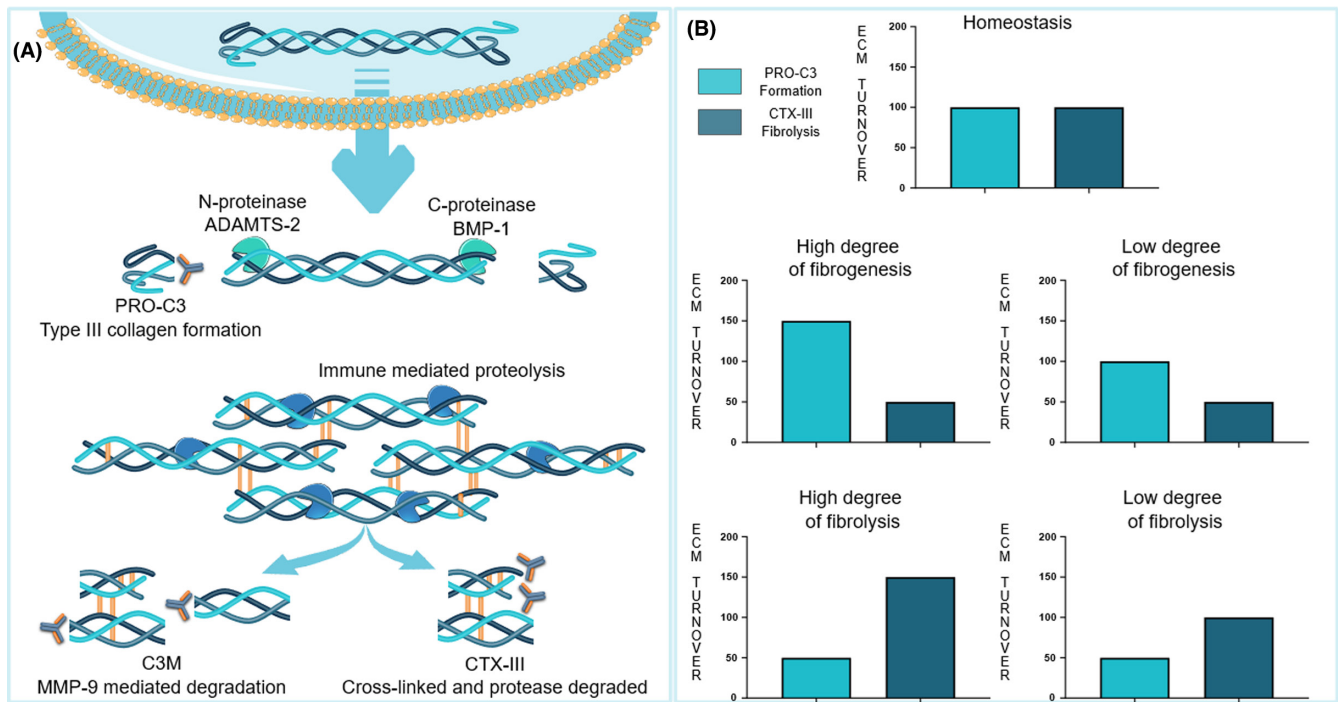


FIGURE 1 The balance between fibrogenesis and fibrolysis. (A) The procollagen is released to the extracellular environment following intracellular collagen α -chain trimerisation, with subsequent proteolytic removal of the pro-peptides by N- and C-proteinases. The collagen monomers are incorporated into the collagen fibrils within the extracellular matrix by forming intra- and inter-molecular enzymatic cross-links. Immune-mediated proteolysis of either newly synthesised collagen monomers or the enzymatically cross-linked collagen multimers results in specific collagen fragments release. Dependent on the protease, formation biomarkers (PRO-C3) or immune-mediated proteolysis are generated (C3M or CTX-III). (B) Measuring type III collagen formation and degradation markers, various endotypes of either fibrogenesis or fibrolysis may be identified

2.2 | Reagents

All reagents used in the experiments were high-quality chemicals from Merck and Sigma. Synthetic peptides used for monoclonal antibody production, assay development, and subsequent validation were 1) Immunogenic peptide: Keyhole Limpet Hemocyanin (KLH)-CGG-KAGGFAPYYG, 2) Coating peptide: Biotin-KAGGFAPYYG, 3) Selection peptide: KAGGFAPYYG or CKAGGFAPYYG x CKAGGFAPYYG (dimer linked by an N-terminal disulphide bridge), 4) Elongated peptide: KAGGFAPYYGD or CKAGGFAPYYGD x CKAGGFAPYYGD (dimer linked by an N-terminal disulphide bridge), 5) Truncated peptide: KAGGFAPYY or CKAGGFAPYY x CKAGGFAPYY (dimer linked by an N-terminal disulphide bridge), and 6) Rat dimeric peptide: CKSGGFSPYYG x CKSGGFSPYYG. The dimeric peptides were only used for assay development and validation. All synthetic peptides were purchased from Genscript.

2.3 | Monoclonal antibody production and clone characterisation

Monoclonal antibodies were generated in Balb/C mice as¹⁹ targeting the neo-epitope (1212'-KAGGFAPYYG-'1221) located in the C-terminal telopeptide of type III collagen. Briefly, 200 μ l of emulsified antigen and 100 μ g immunogenic peptide with Sigma adjuvant

System were injected. We repeated the intravenous injection of immunogenic peptide (100 μ g in 100 μ l 0.9% NaCl) after one month, isolating the splenocytes after 72 h for cell fusion in SP2/0 myeloma cells.²⁷ The supernatants were screened for reactivity against the selection peptide and the elongated peptides in an indirect competitive enzyme-linked immunosorbent assay (ELISA) using streptavidin precoated plates (Roche, Hvidovre, Denmark, cat. No 11940279), coated with 4 ng/ml of the coating peptide. Clones were selected, testing the selective and unique reactivity of antibodies towards the selection peptide or the immunogenic peptide. The elongated and truncated peptides were not detected (Figure 2). The selected antibody was isotyped using the SBA Clonotyping™ System-HRP (Southern Biotech) and purified using a protein-G column from GE Healthcare Life Sciences (Little Chalfont).

The capture antibody was generated by mixing 110 μ l $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 9.6, 1 ml (1 mg/ml) of antibody, and 13.3 μ l of biotinamido hexanoic acid N-hydroxysuccinimide ester and the solution incubated at 20°C for 1 h with end-over-end rotation. Subsequently, 110 μ l of 0.2 M ethanolamine, pH 8.0, was added to the solution and incubated as before. The solution was dialysed overnight in a Zeba 7 k MWCO desalting column (Thermo Scientific, cat No. 89889) submerged in 1x PBS at 4°C. A portion of the solution was labelled with horseradish peroxidase using a kit from Sigma (cat 11829696001) according to the manufacturers' instructions to be used as the detection antibody.

2.4 | CTX-III direct sandwich ELISA protocol

Streptavidin precoated 96-well plates (Roche Diagnostic's, Hvidovre, Denmark, cat. No. 11940279) were coated with 5.8 µg/ml capture antibody diluted 1/100 in assay buffer (50mM PBS, 1% BSA, 0.1% Tween-20, 150mM NaCl, pH 7.4) and incubated 30 min at 20°C rotating 300 rounds-per-minute. Twenty microlitres of standard, control, or sample were added followed by 100 µl of 11.5 µg/ml detection antibody diluted 1/100 in incubation buffer (50mM PBS, 1% BSA, 0.1% Tween-20, 150mM NaCl, 5% Liquid II, pH 7.4). Plates were then placed at 4°C under rotation for 20h. Plates were washed using a 25mM TRIZMA, 50mM NaCl, 0.036% Bronidox L5, and 0.1% Tween 20 buffer between each incubation. Subsequently, 100 µl chemiluminescence substrate was added to the wells, incubated for 3 min at 20°C in the dark, and measured at 450nm and 650nm. With the results obtained from a 2-fold serial dilution of the dimeric selection peptide, a standard curve was plotted using a 4-parametric mathematical fit model. Unknown sample measurements were interpolated with the standard curve to obtain the concentration (ng/ml) of CTX-III.

2.5 | Technical validation

The lower limit of detection (LLOD) was determined from 21 zero samples (i.e. incubation buffer), with the upper limit of detection (ULOD) determined from 10 measurements of the dimeric selection peptide. LLOD and ULOD were calculated as the mean + 3x standard deviation (SD). Inter- and intra-assay variation was determined by ten independent runs of five quality control samples in double determination, with a minimum of three of these being healthy human plasma EDTA samples (Valley Biomedical). The acceptance criteria for inter- and intra-assay variation were 15% and 10% respectively. Assay linearity, specificity, accuracy, and interference were determined by calculating the percentage recovery with a validation criterion of 100% ± 20% from the reference sample. Using the undiluted sample as a reference, we evaluated the linearity by a 1:6-fold dilution of four healthy human plasma EDTA samples. Spiking two healthy human plasma EDTA samples and calculating the recovery between the actual and theoretical measurements determined assay accuracy. Interference was evaluated by spiking healthy human plasma EDTA samples with a known concentration of biotin (low = 15 ng/ml, high 45 ng/ml), haemoglobin (low = 0.078 mM, high = 0.155 mM), or lipid (low = 2.42 mM, high = 5.49 mM). The recovery was calculated between the unspiked and the low or high interferent samples.

2.6 | Analyte and reagent stability

We determined the analyte stability by calculating the percentage recovery of three healthy human plasma EDTA samples from the non-stressed sample. Samples underwent four freeze and thaw cycles or were subjected to 2, 4, 24 or 48 h of incubation at either 4°C or 20°C.

2.7 | Scar-in-a-jar cell model

Human hepatic stellate cells (HSC)s purchased from Zenbio (cat. no. HP-F-S) were grown to confluence and seeded in passage 6–8 at a density of 30000 cells/well in 48-well plates in a high serum medium (10% fetal bovine serum (FBS) (cat. no. F7524, Sigma-Aldrich) in Dulbecco's modified eagle medium (DMEM) + Glutamax (cat. no. 31966, Gibco, Life Technologies)) on day –2. On day –1, the cells were serum-starved in a low serum medium (0.4% FBS DMEM), avoiding interference with biomarker measurements. Upon induction of fibrogenesis on day 0, cells were cultured in a low serum medium containing ficoll-70 (112.5 mg/ml, cat. no. F2878, Sigma-Aldrich) and –400 (75 mg/ml, cat. no. F4375, Sigma-Aldrich), supplemented with 1.0% L-ascorbic acid, phosphate magnesium salt, n-hydrate (cat. no. 013-19641, Wako, Osaka, Japan) without or with stimulation of 20ng/ml TGF-β1 (cat no. 100-B-010/CF, R&D system). Cell culturing occurred by incubation at 37°C with 95% O₂ and 5% CO₂ for 12 days, exchanging for a freshly prepared medium without or with TGF-β1 stimulation on days 0, 4 and 8, saving the supernatants on days 4, 8 and 12 for subsequent PRO-C3 and CTX-III measurements. Three replicates were performed without and with stimulation.

2.8 | Cell-matrix cleavage

On day 12, having removed the supernatant, the scar-in-a-jar (SIAJ) cell model wells were washed twice in PBS and stored at –20°C. For the in vitro cleavages of the matrix-containing wells, the plates were thawed and washed gently with the digestion buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl₂, 100 µM ZnCl, pH 7.5). Pro-forms of MMP-9 (cat. no. 911-MP-010, R&D system) and MMP-13 (cat. no. 511-MM-010, R&D system) were activated by dilution in the digestion buffer to 100ng/µl pro-protease and 1 mM 4-Aminophenylmercuric acetate (APMA, cat. no. A9563, Sigma-Aldrich), incubating pro-MMP-9 for 24 h and pro-MMP-13 for 2 h at 37°C. Five-hundred microlitres of 0.5 µg activated MMP diluted in the digestion buffer, and 35 µg of pepsin (cat. no. P7000, Sigma-Aldrich) diluted in 335 µl 10 mM HCl (pH. 3.0) was added to the wells, incubating the plates at 37°C for 72 h. Subsequently, the proteases were inhibited by adding one µM EDTA to the wells containing MMPs or 135 µl 100 mM NaOH (pH. 11.3) to wells containing pepsin. The cleavage supernatants were stored at –20°C until CTX-III measurements. Wells incubated without proteases only containing the respective digestion buffer were used as a control.

2.9 | Biomarker measurements assay

Assessment of type III collagen formation and degradation was performed using the PRO-C3¹⁸ and C3M¹⁹ competitive ELISAs targeting a neo-epitope in the NH₂-terminal pro-peptide or MMP-9 mediated degradation of the C-terminal helical region respectively. The two ELISAs were manufactured by Nordic Bioscience (Herlev, Denmark)

and performed according to the previously described protocol. All three collagen biomarkers and the standardised liver-relevant biomarker, aspartate aminotransferase (AST), were evaluated in the two clinical cohorts, whilst PRO-C3 and CTX-III were evaluated in the supernatants of the SIAJ cell model on days 4, 8 and 12. CTX-III was evaluated in the supernatants of the cleaved matrix.

2.10 | Statistical analysis

Categorical patient characteristics are presented as frequency (percentage), with continuous variables as mean \pm SD. Statistical differences in categorical patient characteristics were calculated using Fisher's exact test for two groups or the Chi-square test for more than two groups. Differences in continuous variables, including biomarker levels between healthy donors and patients of Cohort 1 and Cohort 2, were done by Mann-Whitney or Kruskal-Wallis applying Dunn's test correcting for multiple comparisons using the non-log-transformed biomarker data. Net fibrolysis is defined as the ratio between fibrolysis (CTX-III) and fibrogenesis (PRO-C3) and calculated by dividing the biomarker levels (CTX-III/PRO-C3). The influence of the patient's body mass index (BMI) was analysed by ANCOVA using the log-transformed biomarker levels as the dependent variable to obtain a non-significant Levene's test for equality of error variances. Biomarker data are depicted as ng/ml or the estimated marginal means from the ANCOVA and plotted as Tukey plots. The biomarker data of the SIAJ cell model was analysed by one-way ANOVA comparing data of non-stimulated and stimulated cells, applying Dunnett's test correcting for multiple comparisons. Data are presented as the mean \pm SD. Statistical analysis of the CTX-III fold-change of the cell-matrix cleavage was done using the exact biomarker measurements (ng/ml) of the three replicates comparing with and without active MMP by one-way ANOVA correcting for multiple comparisons by Šídák. The fold change of CTX-III was calculated based on the respective media controls of the SIAJ and cell-matrix cleavage experiments. All statistical analyses were performed in GraphPad Prism v.9.1.1 (Graph Pad Software) or MedCalc v.19.3 (MedCalc Software). Asterisks indicate the following: * p < .05; ** p < .01; *** p < .001; **** p < .0001; ns = non-significant difference.

3 | RESULTS

3.1 | CTX-III technical performance

No reactivity towards the elongated- or truncated- peptide was observed, reflected by the lack of signal inhibition. The increasing signal inhibition with increasing concentrations of the selection peptide and immunogenic peptide demonstrated antibody specificity towards the neo-epitope (Figure 2). The technical validation, including the analyte and reagent stability, was accepted following the validation criteria. Table 1 summarises these results.

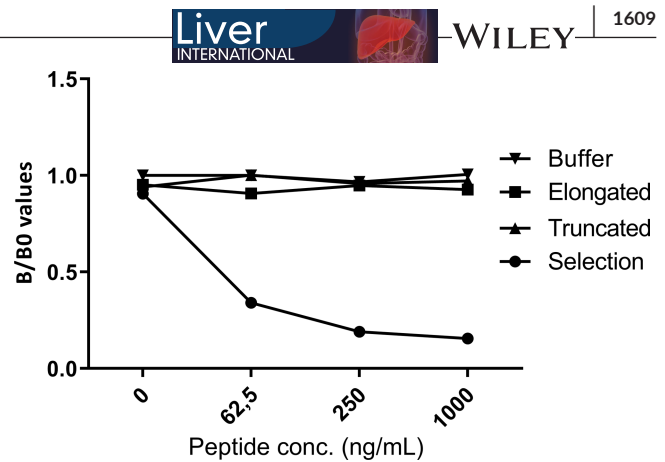


FIGURE 2 Monoclonal antibody specificity. Evaluation of NBH-242 monoclonal antibody specificity in an indirect competitive ELISA. Reactivity towards the selection-, elongated-, truncated-, immunogenic- peptide, and buffer was tested

TABLE 1 Summary of the technical performance and evaluation results

Technical validation procedure	Results
EC ₅₀ (ng/ml)	5.18
LLOD (ng/ml)	0.92
ULOD (ng/ml)	15.94
Inter-assay variation (%)	14.8
Intra-assay variation (%)	5.4
Dilution recovery (range (%))	108.9 (85.6–117.3)
Spiking recovery (range (%))	102.2 (100.8–124.3)
Interference recovery (range (%))	Haemoglobin: 92.7 (103.6–118.1) Biotin: 100.5 (97.2–103.5) Lipid: 100.8 (91.5–111.1)
Freeze–Thaw recovery (range (%))	Up to 4 cycles: 97.7 (83.6–104.9)
Analyte stability recovery (range (%))	Up to 48 h at 4°C: 90.6 (83.8–93.8) Up to 4 h at 20°C: 112.8 (109.7–115.8)

Note: Percentages are reported as mean (range).

Abbreviations: EC₅₀, Effective concentration 50; LLOD, lower limit of detection; ULOD, upper limit of detection.

3.2 | Cohort demographics

Clinical characteristics of the NAFLD (Cohort 1) and HCV (Cohort 2) cohorts are presented in Tables 2 and 3, respectively, displaying the biomarker levels without correcting for BMI. The proportion of patients in Cohort 1 with a NAS score <2 (n = 28/47, 59.6%) at baseline were 40.5% and 38.3% larger than the proportion of patients with a score of 3–4 (n = 9/47, 19.1%) or \geq 5 (n = 10/47, 21.3%). Twice as many patients had a lobular inflammation grade of 0–2 foci/200x

TABLE 2 Schematic description of obese patients with NAFLD before surgery

	NAFLD patients
N	68
Clinical characteristics	
Age (years), mean (SD)	51.6 (10.7)
Gender (female), n (%)	48 (70.6%)
BMI (kg/m ²), mean (SD)	51 (6.8)
NAS score, n (%)	
• <2	28 (59.6%)
• 3–4	9 (19.1%)
• ≥5	10 (21.3%)
Steatosis grade, n (%)	
• <5%	18 (37.5%)
• 5–33%	16 (33.3%)
• >33–66%	8 (16.7%)
• >66%	6 (12.5%)
Lobular inflammation, n (%)	
• No foci	8 (17.8%)
• <2 foci/200x field	22 (48.9%)
• 2–4 foci/200x field	12 (26.7%)
• >4 foci/200x field	3 (6.7%)
Hepatocellular ballooning, n (%)	
• None	30 (65.2%)
• Few ballooned cells	16 (34.8%)
• Many ballooned cells	0 (0%)
Fibrosis stage, n (%)	
• F0	17 (36.2%)
• F1	25 (53.2%)
• F2	5 (10.6%)
• F3	0 (0%)
• F4	0 (0%)
Biomarker levels (ng/ml), mean (SD)	
CTX-III	4.24 (5.2)
PRO-C3	11.34 (6.4)
Net fibrolysis, ratio	0.53 (0.3)
C3M	9.48 (2.0)

Note: Results are presented as mean (SD) or frequency (percentage). Abbreviations: NAFLD, non-alcoholic fatty liver disease; BMI, body mass index.

field ($n = 30/45$, 66.7%) compared with patients with >2 foci/200x field ($n = 15/45$, 33.4%). The majority presented with stage 0 or stage 1 fibrosis ($n = 17/47$, 36.2% and $n = 25/47$, 53.2% respectively), with only 10.6% ($n = 5/47$) having a fibrosis stage of 2. No patients with a fibrosis stage above 2 were present (Table 2).

A total of 82.9% of the population in Cohort 2 had an Ishak stage of 2 or 3 (65/158, 41.1%, and 66/158, 41.28% respectively). At screening, 80% of patients in the Regressors subgroup had Ishak stage 3 ($n = 4/5$), with the remaining 20% having Ishak stage 2 ($n = 1/5$). In the stable group, 60% had Ishak stage 2 ($n = 18/30$), with 26.7% and 13.3% of the patients presenting with Ishak stage 3 ($n = 8/30$) and 4 ($n = 4/30$) respectively. The majority of the Progressors had an initial Ishak stage of 2 ($n = 5/11$, 45.4%) with an equal distribution of patients with Ishak stages 3 and 4 ($n = 3/11$,

27.3%). A statistical difference was observed in AST levels between patients in the Regressors, Stable, and Progressors subgroups ($p < .05$), whereas no difference was observed in the Fibrotest. We observed no statistical difference between the subgroups in age, gender distribution, BMI, ethnicity, or distribution of patients according to Ishak staging (Table 3).

3.3 | Patients with NAFLD or hepatitis C-related hepatic fibrosis present with elevated CTX-III levels

Comparing the measured levels of plasma CTX-III in healthy donors to the patients of Cohort 1 and Cohort 2 demonstrated an elevation in patients presenting with NAFLD or HCV ($p < .0001$, difference of means [NAFLD: 2.48 ng/ml, HepC: 2.55 ng/ml]) (Figure 3).

3.4 | Liver resolution (CTX-III) is elevated in patients with NAFLD undergoing bariatric surgery

The biomarker levels of CTX-III, PRO-C3, net fibrolysis, and C3M were corrected for the baseline BMI of patients with NAFLD. At baseline, plasma CTX-III was significantly elevated compared to the 6-month follow-up ($p < .05$, difference of the estimated marginal means [0.83]) (Figure 4A). Furthermore, PRO-C3 levels were significantly elevated at the 6-month follow-up compared to baseline levels ($p < .05$, [2.52]) (Figure 4B). The calculated degree of net fibrolysis (CTX-III/PRO-C3) resulted in a significant difference between baseline and the 6-months follow-up ($p < .001$, [0.15]) (Figure 4C). Plasma C3M did not differ between groups (Figure 4D).

3.5 | Liver resolution (CTX-III) was not related to the fibrosis stage

The estimated marginal means of each biomarker corrected for BMI were compared between patients presenting with mild (Ishak stage 1–2), moderate (Ishak stage 3), and severe fibrosis (Ishak stage 4–5) at screening. No significant differences between the biomarker levels of CTX-III (Figure 5A), net fibrolysis (Figure 5C), or C3M (Figure 5D) were observed between patient groupings. Plasma PRO-C3 was significantly elevated in severe fibrosis compared to both mild ($p < .0001$, difference of estimated marginal means [15.88]) and moderate fibrosis ($p < .001$, [13.76]) (Figure 5B). Patients with a severe degree of fibrosis presented with elevated AST compared to mild and moderate fibrosis at screening ($p < .001$ [35.54], and $p < .01$ [36.96]).

3.6 | Liver fibrosis resolution (CTX-III) was elevated in regressive patients defined by liver biopsy

Patients in the placebo group were stratified according to a change in Ishak stage from screening to the 52-week follow-up, and the biomarker

TABLE 3 Schematic description of patients with HCV infection

		Regressors	Stable	Progressors	p value
N	158	5	30	11	
Clinical characteristics					
Age (years), mean (SD)	51.6 (6.1)	50.2 (3.9)	52 (6.3)	48.7 (6.0)	NS
Gender (male), n (%)	100 (63.3%)	5 (100%)	20 (66.7%)	7 (63.6%)	NS
BMI (kg/M ²), mean (SD)	28.6 (4.8)	30.9 (4.9)	29.4 (5.0)	31.2 (6.0)	NS
Ethnicity, n (%)					
African American	13 (8.2%)	1 (20%)	0	1 (9.1%)	NS
American Indian or Alaskan native	7 (4.4%)	0	0	1 (9.1%)	
Arabic/North African heritage	2 (1.3%)	0	0	0	
Asian – East Asian heritage	15 (9.5%)	1 (20%)	3 (10%)	1 (9.1%)	
Asian – Southeast Asian heritage	5 (3.2%)	0	1 (3.3%)	0	
Caucasian	116 (73.4%)	3 (60%)	26 (86.7%)	8 (72.7%)	
Fibrosis stage, n (%)					
Ishak stage 1	2 (1.3%)	0	0	0	NS
Ishak stage 2	65 (41.1%)	1 (20%)	18 (60%)	5 (45.4%)	
Ishak stage 3	66 (41.8%)	4 (80%)	8 (26.7%)	3 (27.3%)	
Ishak stage 4	23 (14.6%)	0	4 (13.3%)	3 (27.3%)	
Ishak stage 5	2 (1.3%)	0	0	0	
Change in Ishak stage, mean	NA	-1	0	1	
Biochemical variables, mean (SD)					
ALT	72.7 (47.0)	ND	ND	ND	
AST	63.9 (40.1)	42.6 (21.4)	56.5 (40.4)	112.2 (72.1)	<0.05 ^a
Fibrotest	0.6 (0.2)	0.6 (0.2)	0.6 (0.3)	0.7 (0.2)	NS
Biomarker levels (ng/ml), mean (SD)					
CTX-III	4.20 (3.5)	6.34 (5.5)	3.36 (0.9)	3.03 (1.8)	<0.05 ^a
PRO-C3	20.17 (12.9)	14.58 (5.2)	19.43 (11.9)	32.55 (14.2)	<0.05 ^a
Net fibrolysis (ratio)	0.45 (0.2)	0.61 (0.3)	0.42 (0.1)	0.30 (0.1)	<0.01 ^a
C3M	4.71 (1.5)	3.98 (1.4)	4.75 (1.9)	5.07 (1.9)	NS ^a

Note: Results are presented as mean (SD) or frequency (percentage). Statistical differences were calculated using one-way ANOVA or chi-square test. Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; NA, not applicable; ND, not determined; NS, not significant.

^aBased on biomarker levels uncorrected for BMI, calculated by Kruskal-Wallis applying Dunnett's test for multiple comparisons.

data corrected for patient BMI at screening. Plasma CTX-III at screening significantly differentiated a spontaneous regressive endotype of fibrosis from patients with a stable or progressive endotype ($p < .05$, difference of estimated marginal means [3.79] and $p < .01$ [4.12]) (Figure 6A). Levels of PRO-C3 were significantly elevated in patients with a progressive endotype compared to patients with a stable ($p < .01$ [13.12]) or regressive endotype ($p < .01$ [17.97]) (Figure 6B) respectively. The degree of net fibrolysis at screening allowed a significant differentiation between a regressive and stable endotype ($p < .05$ [0.32]) and a regressive and progressive endotype ($p < .001$ [0.41]) (Figure 6C). Plasma C3M could not differentiate between the three endotypes (Figure 6D). The standardised biomarker, AST, differentiated patients with a progressive fibrotic endotype from patients with a regressive or stable endotype ($p < .05$ [68.30], and $p < .01$ [55.96]) (Figure 6E).

3.7 | TGF- β 1-induced PRO-C3 release and MMP-catalysed release of CTX-III in an in vitro model of hepatic fibrosis

A proof of concept experiment was conducted to demonstrate whether PRO-C3 is indeed a marker of fibrogenesis and CTX-III a marker of fibrolysis. Fibrosis was induced in HSCs using TGF- β 1 for 12 days with subsequent proteolytic degradation of the deposited collagen matrix. At day 4, 8, and 12 the PRO-C3 levels measured in the supernatants of HSCs stimulated with 20-ng/ml (day 4: $p < .001$, difference of means [34.12 ng/ml], day 8: $p < .5$ [51.56 ng/ml], and day 12: $p > 0.05$ [109.25 ng/ml]) of TGF- β 1 were elevated compared to cells without stimulation (Figure 7A). Measuring CTX-III in the supernatant resulted in no significant release of the biomarker on days

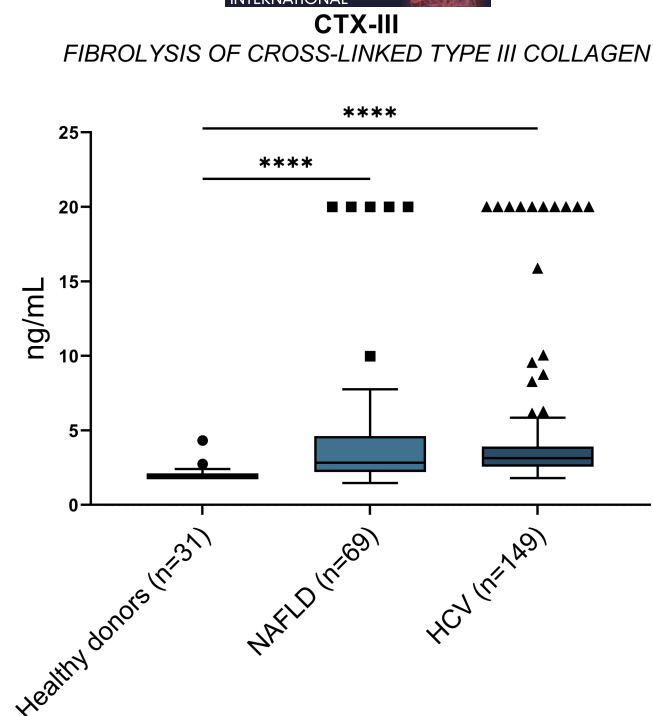


FIGURE 3 The biological relevance of the CTX-III biomarker. Plasma CTX-III was plotted for healthy donors and patients with NAFLD (Cohort 1) or HCV (Cohort 2). The data is presented as a Tukey plot calculating the statistical difference between the healthy donors and NAFLD or HepC by Kruskal-Wallis using Dunn's to correct for multiple comparisons. The statistical significance is depicted as **** $p < .0001$

4, 8 and 12 (Figure 7B). Cleaving the SIAJ cell-matrix with MMP-9 or -13 resulted in a significant release of CTX-III compared to the media control ($p < .0001$ and $p < .0001$ respectively) (Figure 7C).

4 | DISCUSSION

In the present study, we sought to investigate the clinical relevance of the CTX-III biomarker, which quantifies cross-linked proteolytic fragments of type III collagen for the assessment of fibrosis resolution. Our data indicated the following: (1) A technically robust sandwich ELISA capable of quantifying cross-linked type III collagen proteolysis. (2) Obese patients with NAFLD (Cohort 1) and patients with HCV-associated liver fibrosis (Cohort 2) demonstrated elevated plasma CTX-III compared to healthy donors demonstrating the biological relevance of the biomarker. (3) Plasma CTX-III at the screening in the placebo group of Cohort 2 enabled differentiation between spontaneous regressors of fibrosis from patients with stable or progressive fibrosis. (4) In an *in vitro* model, pro-fibrotic stimulation resulted in type III collagen deposition quantified by PRO-C3, whereas the subsequent degradation of type III collagen by MMP-9 and -13 resulted in the release of the CTX-III biomarker. By including the PRO-C3 and C3M biomarkers reflecting formation and MMP-9 mediated degradation of type III collagen, respectively, we evaluated

the importance of targeting the "right" neo-epitope of type III collagen biomarkers.

4.1 | CTX-III is associated with fibrolysis in NAFLD patients

The significant deposition of type III collagen is well-known in liver fibrosis, which, combined with extensive lysine hydroxylation and subsequent lysine oxidase (like) enzyme catalysed cross-linking, results in a stiff ECM perpetuating fibrosis progression.¹⁶ The extensive enzymatic cross-linking of the collagens decreases protease-mediated collagen catabolism, thereby increasing collagen accumulation.^{28,29} There is an increasing focus on developing pro-resolution therapy, which includes inhibitors of collagen cross-linking enzymes, such as LOXL2.³⁰ *In vitro* data of lysyl oxidase inhibition demonstrated increased collagen resolution with fibrotic resolution.³¹

In the clinical assessment of liver fibrosis, liver biopsies remain the reference standard. However, tissue biopsies are invasive, associated with morbidity and mortality, and sampling and pathology scoring discordance between samples obtained from the same patient.^{5,6,32} Though several non-invasive serological biomarkers are available for cross-sectional assessment of the fibrosis stage, none relate directly to the accumulation or resolution of the hepatic collagen content or reflect the balance between fibrogenesis and fibrolysis.^{33,34}

We initially evaluated biomarkers of type III collagen remodelling in NAFLD. NAFLD is one of the major chronic liver diseases affecting about one-quarter of the population,⁹ characterised by excessive hepatic fat accumulation via lipid intake (obesity), resulting in inflammation and fibrosis.⁸ Previous studies of Cohort 1 included in the present study demonstrated metabolic alterations,³⁵ changes in lipoproteins²³ and inflammatory improvement following bariatric surgery.³⁶ Herein, we demonstrated increased plasma CTX-III in patients following their pre-operative weight loss. Quantifying cross-linked and protease degraded type III collagen fragments, the levels of CTX-III indicate an elevation of fibrolysis and, potentially, fibrosis resolution.

In contrast, type III collagen formation (PRO-C3) and type III collagen resolution (CTX-III) were inversely associated at baseline and the 6-month follow-up. The pre-operative weight loss and potential alterations of liver functions, including inflammation, may induce proteolytic resolution of accumulated and cross-linked fibrotic collagen,³⁷ potentially observed as increased plasma CTX-III. With the recession of inflammation, new type III collagen is synthesised, restoring tissue integrity. Note that approx. 33% of the patients presented with ≥ 2 foci/200x field at baseline (Table 2). Whilst previous studies indicate fibrosis regression following bariatric surgery with collagen deposition and degradation changes,^{38,39} the lack of a second follow-up biopsy and significant histological fibrosis limits our final interpretation of the association between fibrosis regression and this study's fibrolytic and

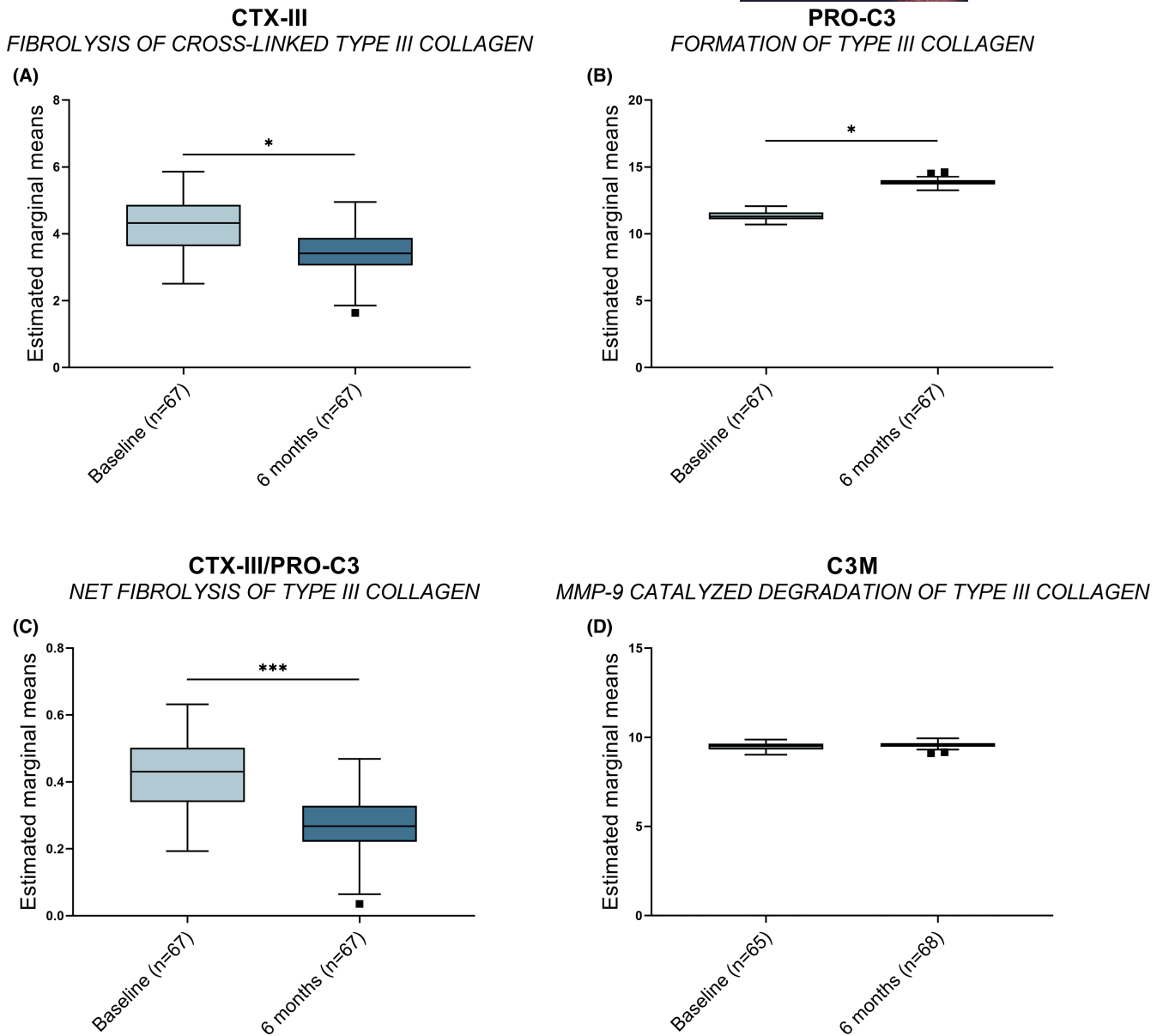


FIGURE 4 Bariatric surgery modifies circulating biomarkers. The estimated marginal means of CTX-III (A), PRO-C3 (B), net fibrolysis (C), and C3M (D) at baseline and the 6-month follow-up in patients with NAFLD. The estimated marginal means were obtained by ANCOVA using patient BMI at baseline as a covariate. Data is presented as a Tukey plot calculating the statistical difference between baseline and the 6-month follow-up by Mann-Whitney. The statistical significance is depicted as * $p < .05$, and *** $p < .001$

fibrogenic biomarkers. Though 36.7% of the patients presented with F0 at baseline (Table 2), the sampling variability of the liver biopsy may underestimate the degree of fibrotic ECM. Thus, the elevated degree of fibrolysis observed may still reflect fibrosis resolution, albeit the current degree of histological fibrosis is subclinical.

4.2 | CTX-III as a prognostic marker of liver fibrosis development

In addition to hepatic lipid accumulation, viral infections such as HCV are a common underlying cause of progression to liver fibrosis.

Treatment of HCV with highly effective direct-acting antiviral drugs has recently led to universal sustained virologic response (SVR).⁴⁰ Removal of the underlying cause of tissue injury has resulted in fibrosis regression in both HCV³³ and other hepatic pathologies.³³ However, the exact molecular mechanisms have yet to be fully elucidated, partially caused by the rarely performed liver biopsy following SVR.³³ We used the biopsy-dependent linear Ishak staging system²⁶ to depict liver fibrosis. Assessing baseline levels of type III collagen remodelling according to the Ishak staging of liver fibrosis, we evaluated the diagnostic value of the biomarkers. Whilst the CTX-III biomarker demonstrated no diagnostic value of the CTX-III biomarker, we observed an increasing degree of fibrogenesis quantified by PRO-C3 in advanced stages of fibrosis. The ability of PRO-C3

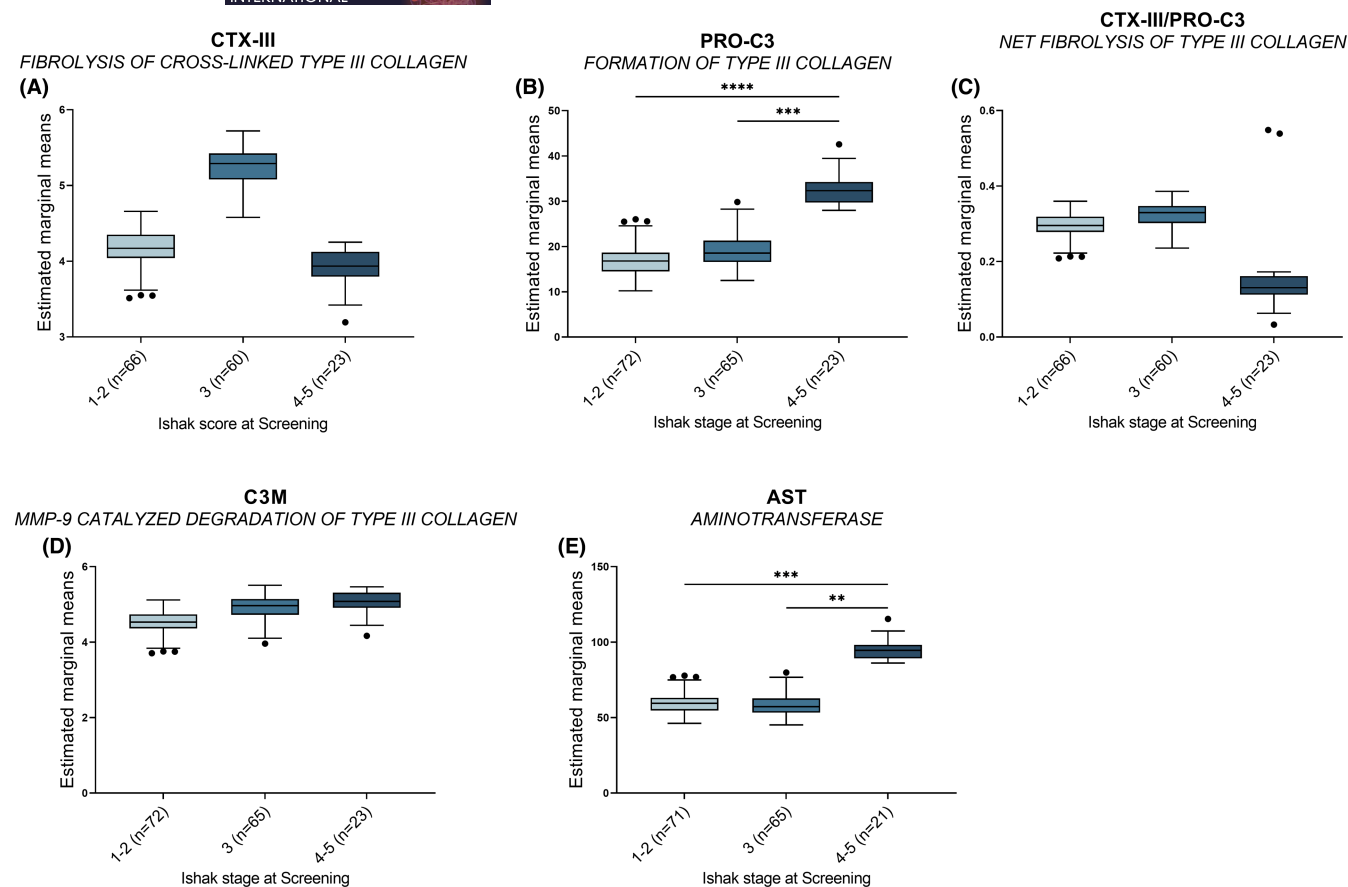


FIGURE 5 Fibrosis severity is associated with fibrogenesis. The estimated marginal means of CTX-III (A), PRO-C3 (B), net fibrolysis (C), and C3M (D) was plotted for the Ishak stage at the initial histological screening of patients with HCV-associated liver fibrosis. The estimated marginal means were obtained by ANCOVA using patient BMI at screening as a covariate. Data is presented as a Tukey plot calculating the statistical difference between the stages by Kruskal-Wallis using Dunn's test to correct for multiple comparisons. The statistical significance is depicted as: ** $p < .01$, *** $p < .001$, and **** $p < .0001$

to differentiate severe liver fibrosis from mild and moderate fibrosis has been demonstrated previously.⁴¹ Our data indicate that the PRO-C3 biomarker rather than CTX-III could aid in assessing hepatic fibrosis severity and fibroblast activity, emphasizing the quantification of fibrogenesis in diagnosing hepatic fibrosis.

The prognostic value of the biomarkers was evaluated by correlating the biomarker levels at the initial screening with their fibrotic endotype. Achieving fibrosis resolution requires a higher degree of fibrolysis than fibrogenesis, thus resulting in a net surplus of collagen degradation, measurable by the CTX-III biomarker. We demonstrated how cross-linked proteolytic degradation fragments of type III collagen were associated with regressive fibrosis and formation associated with progressive fibrosis. In line with previously published data, type III collagen formation at the screening was elevated in patients presenting a progressive endotype.⁴² Compared to Cohort 1, the patients of Cohort 2 presented with significant histological fibrosis, strengthening our assumption of the CTX-III biomarker relating to fibrolysis and potentially fibrosis regression. As CTX-III and PRO-C3 represent each side of type III collagen turnover, fibrolysis, and fibrogenesis, respectively, we calculated the net fibrolysis based on both biomarker levels. Using the net fibrolysis enhanced

the statistical differentiation between regressors and progressors of fibrosis. For both the diagnostic and prognostic biomarker potential of CTX-III and PRO-C3, we compared with the commonly used AST biomarker. As a diagnostic biomarker, AST outperformed CTX-III, with comparable potential to PRO-C3. However, as a biomarker of fibrosis regression, the ratio of CTX-III and PRO-C3 outperformed AST in this study, indicating the potential of combining multiple biomarkers of collagen remodelling. Thus, by measuring CTX-III and PRO-C3 at the initial histological evaluation, the ratio between the two biomarkers could potentially aid in predicting a regressive- (high fibrolysis/low fibrogenesis) or progressive- fibrotic endotype (low fibrolysis/high fibrogenesis).

Additionally, Karsdal et al.⁴³ demonstrated an association between a regressive endotype and reduced type V collagen formation. Type V collagen acts as auxiliary fibrillar collagen, regulating collagen fibril formation and size. Combined with the data presented here, using biomarkers of protease degraded and cross-linked type III collagen and type V collagen formation could aid in assessing fibrosis regression, presented as a high degree of type III collagen fibrolysis and low degree of type III and type V collagen formation. As fibrosis regression is probably not linear, the use of non-invasive

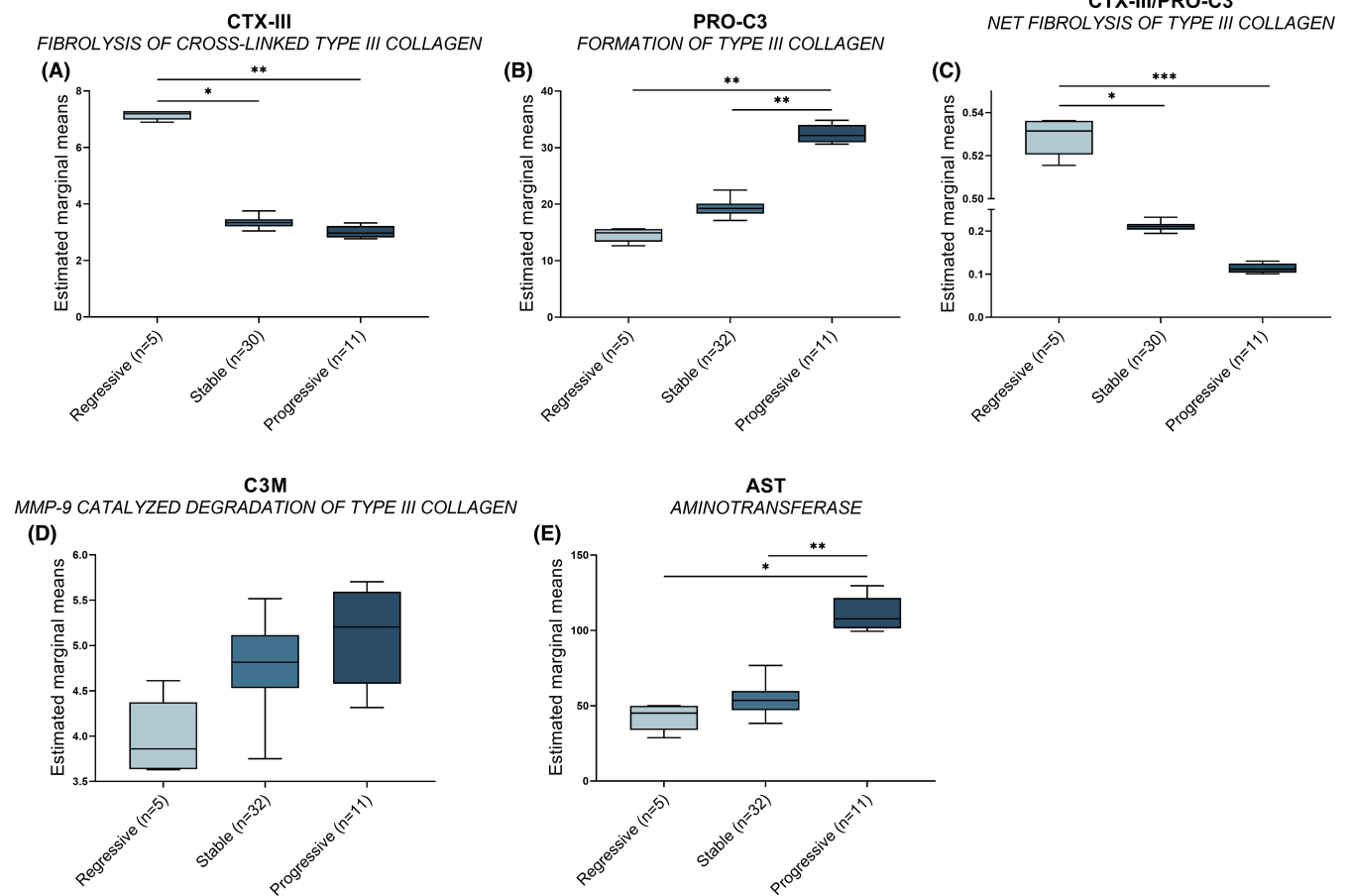


FIGURE 6 Fibrogenetic and fibrolytic biomarkers differentiate endotypes. The estimated marginal means of CTX-III (A), PRO-C3 (B), net fibrolysis (C), and C3M (D) was plotted for the three spontaneous endotypes: regressive, stable, and progressive. The estimated marginal means were obtained by ANCOVA using patient BMI at screening as a covariate. Data is presented as a Tukey plot calculating the statistical difference between the stages by Kruskal-Wallis using Dunn's test to correct for multiple comparisons. The statistical significance is depicted as * $p < .05$, ** $p < .01$, and *** $p < .001$

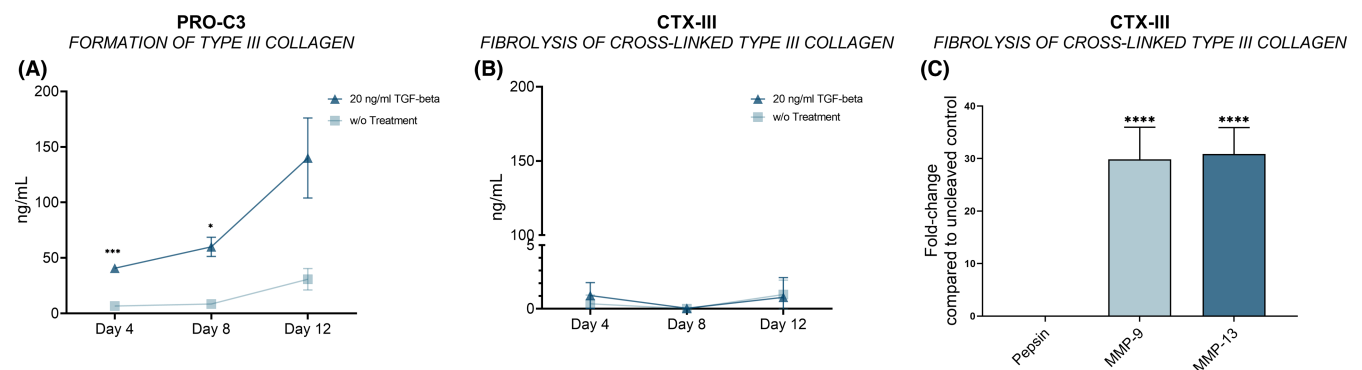


FIGURE 7 In vitro formation and proteolytic degradation of type III collagen. (A) Depicts the measured PRO-C3 levels at day 4, 8, and 12 in the supernatant of the SIAJ cell model without or with 20 ng/ml of TGF- β 1 stimulation. (B) The CTX-III levels measured in the SIAJ supernatants are plotted for days 4, 8, and 12 for HSCs without or with 20 ng/ml of TGF- β 1 stimulation. (C) The fold-change of CTX-III levels was calculated from the uncleaved media control measured in the supernatants following pepsin, MMP-9, or MMP-13 cleavage of the HSC matrix at day 12. The statistical significance was calculated by two-way-ANOVA applying Dunnett's correcting for multiple comparisons (A and B), or one-way ANOVA applying Sidak correcting for multiple comparisons (C) and depicted as * $p < .05$, ** $p < .01$, *** $p < .001$, and **** $p < .0001$

biomarkers capable of quantifying both fibrolysis and fibrogenesis could provide a more optimal assessment of the underlying molecular processes, potentially improving personal therapy. Although

we observed histological regression in Cohort 2, only 5/46 patients regressed, which is because of the inherent sampling variability of the liver biopsy may question the actual degree of regression.^{6,44}

Moreover, the follow-up period of 52 weeks may be too short to assess fibrosis regression fully, as previous studies demonstrate fibrosis regression range from 12 months to 36 months³³ and references therein).

As a proof of concept, we investigated PRO-C3 and CTX-III in a SIAJ cell model using HSCs to mimic hepatic fibrosis. We expected PRO-C3 to be a type III collagen formation marker reflecting fibrogenesis, whereas CTX-III would be released with increasing proteolytic activity reflecting fibrolysis. The stimulation with TGF- β 1, a classical pro-fibrotic growth factor, induced the release of PRO-C3 in the cell supernatants but did not result in the release of CTX-III. In contrast, CTX-III could only be detected following MMP-9 or MMP-13 induced proteolysis of the cell matrix. MMP-9 and MMP-13 have been directly or indirectly linked to collagen degradation in liver fibrosis, potentially driving macrophage-mediated resolution of the fibrotic ECM.⁴⁵ The data of the fibrotic in vitro model supports the notion of PRO-C3 potentially quantifying the build-up of fibrotic tissue, whereas CTX-III quantifies its proteolytic degradation following enzymatic cross-linking (Figure 1). Moreover, our use of neo-epitope specific biomarkers of fibrogenesis and fibrolysis quantifying type III collagen remodelling by targeting specific post-translational modifications can provide a more accurate assessment compared to full-length protein quantifications.

Collectively, the clinical and pre-clinical data presented indicate a potential of CTX-III and PRO-C3 as non-invasive biomarkers applicable for the evaluation of fibrosis regression and progression. However, the final interpretation should be carefully considered in light of the limitations, requiring further validation in clinical studies to redeem the current limitations.

5 | CONCLUSION

We demonstrated a potential clinical use of a technically stable neo-epitope-specific ELISA (CTX-III). By quantifying protease degraded fragments of cross-linked type III collagen in obese patients with NAFLD programmed for bariatric surgery and patients with HCV-associated liver fibrosis, the CTX-III biomarker demonstrated relevance as a disease marker. Furthermore, the CTX-III biomarker alone or in combination with PRO-C3 differentiated patients with a spontaneous regressive or progressive fibrotic endotype. Collectively these data demonstrate the potential of the CTX-III biomarker as a marker of fibrolysis, which may distinguish individuals with varying degrees of active fibrolysis, potentially allowing for a novel non-invasive prognostic tool.

ACKNOWLEDGEMENTS

The Danish Research Foundation supported the development and assessment of the ECM biomarkers.

CONFLICT OF INTEREST

Martin Pehrsson, Shu Sun, Ida Falk Villesen, Mette Juul Nielsen and Joachim Høg Mortensen are employed at Nordic Bioscience

A/S, a company involved in the discovery and development of biochemical biomarkers. Tina Manon-Jensen, Anne-Christine Bay-Jensen, Diana Julie Leeming and Morten Asser Karsdal are employed at and own stocks in Nordic Bioscience A/S. Helena Castañé, Jorge Joven, Keyur Patel and Zachary Goodman have no competing interests.

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How to cite this article: Pehrsson M, Manon-Jensen T, Sun S, et al. An MMP-degraded and cross-linked fragment of type III collagen as a non-invasive biomarker of hepatic fibrosis resolution. *Liver Int.* 2022;42:1605-1617. doi: [10.1111/liv.15270](https://doi.org/10.1111/liv.15270)