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Permalink

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Journal

Alimentary Pharmacology & Therapeutics, 42(5)

ISSN

0269-2813

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Publication Date

2015-09-01

DOI

10.1111/apt.13292

Peer reviewed



Published in final edited form as:

Aliment Pharmacol Ther. 2015 September ; 42(5): 582–590. doi:10.1111/apt.13292.

Novel association between serum pentraxin-2 levels and advanced fibrosis in well-characterised patients with non-alcoholic fatty liver disease

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SUMMARY

Background—Pentraxin-2 (PTX-2), a serum protein, inhibits inflammation and fibrosis, and recombinant PTX-2 is being tested as an anti-fibrotic agent.

Aim—To evaluate the association between serum PTX-2 levels and fibrosis stage in patients with non-alcoholic fatty liver disease (NAFLD).

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AUTHORSHIP

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Author contributions: Elizabeth C. Verna made a substantial contribution to research design, the analysis and interpretation of data, drafted and revised the manuscript and approved the submitted and final version. Janki Patel made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. Ricki Bettencourt made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. Phirum Nguyen made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. Carloyn Hernandez made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. Mark A Valasek made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. Tatiana Kisselva made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. David A. Brenner made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version. Rohit Loomba made a substantial contribution to research design, the analysis and interpretation of data, revised the manuscript and approved the submitted and final version.

All authors approved the final version of the article, including the authorship list.

Methods—Serum pentraxin-2 levels were compared between four groups of well-characterised patients including NAFLD with no fibrosis, NAFLD with mild-moderate fibrosis (stage 1–2), NAFLD with advanced fibrosis (stage 3–4), and age-sex matched non-NAFLD controls.

Results—Sixty subjects were included in the study. The mean age was 58.9 years, 68% were male and 58% were Caucasian. In univariate analysis, serum PTX-2 levels significantly decreased from non-NAFLD controls to mild NAFLD with no fibrosis, to NAFLD with mild-moderate fibrosis and were lowest in patients with NAFLD and advanced fibrosis, in a dose-dependent manner ($P < 0.0001$). In multivariable-adjusted analyses controlling for age, sex, albumin, and CRP, the results remained consistent and statistically significant. Serum PTX-2 level had an AUROC of 0.84 (95% CI: 0.71–0.97) for the diagnosis of NAFLD, and an AUROC of 0.77 (95% CI: 0.65–0.90) for the diagnosis of advanced fibrosis in NAFLD. Serum PTX-2 levels also decreased with increasing liver stiffness as estimated by magnetic resonance elastography ($r = -0.31$, $P = 0.02$).

Conclusions—PTX-2 levels are significantly lower in patients with NAFLD compared to non-NAFLD controls, and decline further in patients with advanced fibrosis. PTX-2 may therefore be both a biomarker of disease and a potential target for anti-fibrotic therapy with the recombinant pentraxin-2.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the United States, yet effective diagnostic and therapeutic strategies are notably lacking.¹ The absence of accurate noninvasive markers to differentiate simple steatosis from more clinically significant forms of NAFLD including non-alcohol steatohepatitis (NASH),² and the limited availability of techniques such as ultrasound elastography to identify patients with NAFLD-related fibrosis, render the development of novel diagnostic and treatment strategies urgent.

Pentraxins are a family of highly conserved serum proteins with diverse functions in innate immunity, as acute phase reactants, and in the regulation of responses to cellular damage and fibrotic remodelling. Pentraxin-2 (PTX-2) has been shown to modify neutrophil adhesion,^{3, 4} inhibit fibrocyte differentiation,^{4–8} and enhance phagocytosis of cell debris by phagocytes and macrophages.^{4, 9, 10} Thus, PTX-2 plays an important role in inhibiting multiple aspects of the regulation of fibrogenesis and wound healing.¹¹ Serum PTX-2 levels also correlate inversely with lung function in models of pulmonary fibrosis,^{12, 13} and maintenance of PTX-2 levels at a site of inflammation and injury may prevent fibrosis formation and progression.

Administration of exogenous PTX-2 has thus been tested in animal models in several forms of chronic fibrotic disease, including carbon tetrachloride (CCl₄) and bile duct ligation-induced liver fibrosis, leading to diminished fibrosis progression.¹⁴ Recombinant PTX-2 is also now being administered in human clinical trials for the prevention and treatment of chronic fibrotic diseases. In the first report of recombinant PTX-2 (PRM-151) in healthy volunteers and patients with pulmonary fibrosis, baseline PTX-2 levels were lower in those patients with pulmonary fibrosis compared with controls, and PTX-2 infusion resulted in a

decrease in the percentage of circulating fibrocytes.¹³ As a result, larger clinical trials are now ongoing in patients with pulmonary fibrosis, as well as myelofibrosis and for the prevention of scarring following trabeculectomy for glaucoma.¹⁵

PTX-2 may therefore be both a biomarker of a profibrotic response to tissue injury, as well as a therapeutic target for fibrotic disease states. We aimed for the first time to evaluate the association between serum PTX-2 levels and fibrosis stage in patients with NAFLD. We hypothesised that serum PTX-2 levels would be inversely associated with hepatic fibrosis as replacement of serum PTX-2 is considered a potential and novel anti-fibrotic strategy for the treatment of hepatic fibrosis in NAFLD.

MATERIALS AND METHODS

Setting, study design and participants

This is a *cross-sectional* analysis of uniquely well-characterised adult patients with NAFLD enrolled in a previously reported prospective cohort.^{15, 16} Patients in this cohort underwent a standardised clinical research visit including history, physical exam, biochemical phenotyping, liver biopsy, magnetic resonance imaging-estimated proton density fat fraction (MRI-PDFF) for fat quantification and magnetic resonance elastography (MRE) to measure liver stiffness. Patients with NAFLD were selected, matched for age and gender, from three well-characterised groups: NAFLD with no fibrosis (stage 0), NAFLD with mild-moderate fibrosis (stage 1–2) and NAFLD with advanced fibrosis (stage 3–4). In addition, non-NAFLD controls were also evaluated, again matched for age and gender. All subjects in this study were recruited from the UCSF NAFLD Translational Research Unit and signed informed consent. This study was approved by the Institutional Review Board.

NAFLD patient cohort.—All patients with NAFLD in this study had liver biopsy-proven disease and an MRI-PDFF fat fraction $\geq 5\%$. Inclusion Criteria for the NAFLD cohort included (i) age at least 18 years, (ii) ability and willingness to give written, informed consent and (iii) minimal or no alcohol use history consistent with NAFLD. Exclusion Criteria included (i) clinical or histological evidence of alcoholic liver disease, (ii) regular and excessive use of alcohol within the 2 years prior to interview defined as alcohol intake greater than 14 drinks per week in a man or greater than 7 drinks per week in a woman, or (iii) secondary causes of hepatic steatosis including previous surgeries, bariatric surgery, total parenteral nutrition, short bowel syndrome, steatogenic medications, evidence of chronic hepatitis B as marked by the presence of HBsAg in serum, evidence of chronic hepatitis C as marked by the presence of anti-HCV or HCV RNA in serum, evidence of other causes of liver disease, such as alpha-1-antitrypsin deficiency, Wilson's disease, glycogen storage disease, dysbetalipoproteinemia, haemochromatosis, autoimmune liver disease, or drug-induced liver injury or concomitant severe underlying systemic illness that in the opinion of the investigator would interfere with the study.

Non-NAFLD healthy controls.—Non-NAFLD healthy controls with no clinical or biochemical evidence of liver disease and $<5\%$ fat fraction by MRI-PDFF were selected from a second prospective cohort of patients with NAFLD and their unaffected family members (NCT01643512).

Inclusion criteria in the non-NAFLD control group included (i) age greater than 18 years; (ii) liver MRI-PDFF <5% and (iii) no history of known liver disease. Exclusion criteria included (i) age less than 18 years; (ii) significant systemic illness; (iii) inability to undergo MRI and (iv) evidence of possible liver disease, including any previous liver biopsy, positive hepatitis B surface antigen, hepatitis C viral RNA, or autoimmune serologies, alpha-1 antitrypsin deficiency, haemochromatosis genetic testing or low ceruloplasmin.

Histological assessment

All patients with NAFLD underwent liver biopsy, which was evaluated by an experienced liver pathologist blinded to clinical, radiology and biomarker data. All biopsies were scored using the NASH clinical research network histological scoring system,¹⁶ including fibrosis stage (range 0–4). NAFLD patients were classified as having NAFLD with no fibrosis (stage 0), NAFLD with mild-moderate fibrosis (stage 1–2) and NAFLD with advanced fibrosis (bridging fibrosis or cirrhosis, stage 3–4). In addition, the NAFLD Activity Score (NAS), and its component measures including the severity of steatosis (range 0–3), inflammation (range 0–3) and hepatocyte ballooning (range 0–2) were evaluated.

Magnetic resonance imaging and elastography assessment

MRI-PDFF fat quantification was available for all patients. As liver biopsy is unethical in normal individuals and other non-invasive measures such as ultrasound and computed tomography lack sufficient sensitivity to exclude steatosis, especially at liver fat fraction between 1% and 10%, MRI-PDFF was utilised for fat quantification and for the exclusion of steatosis in the non-NAFLD control group. MRI-PDFF is highly accurate, sensitive, reproducible and precise in the identification of hepatic steatosis.^{17, 18} A detailed description of the MRI-PDFF protocol has been previously published.^{17–23}

MRE-derived liver stiffness was also measured for all patients. Previous studies have shown that MRE is an accurate and robust, non-invasive, quantitative imaging biomarker for hepatic fibrosis.^{23–25} MRE exams were performed with patients in the supine position, with a 19-cm diameter acoustic pressure-activated passive driver placed over the right anterior chest wall with its center level with xiphoid process. Two-dimensional (2D) gradient echo MRE acquisitions were performed with parameters as previously described.^{23, 24, 26–29} The data were processed to generate images depicting the complex shear stiffness of liver tissue, using direct inversion.^{26, 27} The elastograms were transferred offline for analysis with a trained image analyst who manually drew regions of interest (ROIs) on the elastograms. ROIs were drawn at each of the four slice locations in portions of the liver in which the corresponding wave images showed clearly observable wave propagation. The mean liver stiffness was calculated by averaging the per-pixel stiffness values across the ROIs at the four slice locations.

All MRI-PDFF and MRE measurements were made in the analyses of the original patients cohorts, blinded to histological and clinical data, and prior to PTX-2 measurement.

Pentraxin-2 measurement

A fasting serum sample was collected in the morning of the clinical research visit and stored in a -80°C freezer. The median (IQR) time between blood sample collection and liver biopsy was 35 (86) days and between blood and MRE exam as 11 (35.5) days. Serum PTX-2 and C-reactive protein (CRP, another member of the Pentraxin protein family) levels were assayed by ELISA (Ray Biotech, Norcross, GA, USA). All samples were run in triplicate, blinded to the group assignment and clinical status of the patient. All blood tests were performed on blood sampled from a single day.

Statistical analysis and sample-size estimation

Baseline characteristics and biomarker levels were compared across the four groups by ANOVA or Fischer's exact test as appropriate. Receiver operating characteristic (ROC) curve analysis was performed for PTX-2 as a classifier of advanced fibrosis, as well as of NAFLD as compared to non-NAFLD controls. The measure of overall performance was the area under the ROC (AU-ROC). PTX-2 levels were also correlated with liver stiffness by two-dimensional MRE.

Power analysis indicated a 81% chance of detecting an effect size of a 1.2 standard deviation difference overall between the highest and lowest group means as significant at an alpha of 0.05 (two-tailed) with a total of 60 patients distributed equally between the four groups. All statistical analyses were performed using SAS 9.3 (Cary, NC, USA).

RESULTS

Baseline characteristics

A total of 60 patients are included in this study, 15 in each of four groups: non-NAFLD controls, NAFLD with no fibrosis, NAFLD with mild-moderate fibrosis, and NAFLD with advanced fibrosis. Demographical characteristics were similar between groups, including mean age, race and ethnicity (Table 1). Patients with NAFLD and moderate or advanced fibrosis were more likely to have diabetes mellitus, and had higher glucose levels, haemoglobin A1c and body mass index (BMI) compared to those with mild NAFLD or normal controls. In addition, patients with NAFLD had statistically significant elevations in aminotransferases compared to non-NAFLD controls, and those with advanced fibrosis had significantly lower platelets than patients in other groups. CRP levels did not differ between groups.

Association between serum PTX-2 levels and stage of fibrosis.—Mean (95% confidence interval) PTX-2 levels were significantly lower in patients with advanced fibrosis [12.2 (−4.3–28.7) $\mu\text{g/mL}$] compared to those with mild-moderate fibrosis [22.0 (5.5–38.6) $\mu\text{g/mL}$], NAFLD with no fibrosis [23.7 (7.2–40.2) $\mu\text{g/mL}$] and non-NAFLD controls [80.2 (63.7–96.7) $\mu\text{g/mL}$] (Table 1, Figure 1).

When adjusted in multivariable analysis for age and gender, with or without the addition of either albumin or CRP, these relationships were similar and remained significant with $P < 0.001$ (Table 1).

PTX-2 level had an AUROC of 0.77 (95% CI: 0.65–0.90) for the identification of patients with advanced fibrosis (Figure 2a). A PTX –2 level of 14.7 µg/mL had a positive and negative predictive value for advanced fibrosis of 44% and 87% respectively.

Association between serum PTX-2 levels and presence of NAFLD.—Mean (95% confidence interval) serum PTX-2 levels were significantly lower in patients with NAFLD and any stage of fibrosis compared to normal controls [19.3 (14.6–24.0) µg/mL vs. 80.2 (48.0–112.5) µg/mL, $P = 0.001$].

PTX-2 had an AUROC of 0.84 (95% CI: 0.71–0.97) for the identification of NAFLD compared to normal controls (Figure 2b). A PTX –2 level of 24 µg/mL had a positive and negative predictive value for NAFLD of 92% and 55% respectively.

ALT was significantly negatively correlated with PTX-2 level ($r = -0.33$, $P = 0.01$), while BMI ($r = -0.19$, $P = 0.15$), and haemoglobin A1c ($r = -0.06$, $P = 0.67$) were not significantly correlated with PTX-2 level.

Association between serum PTX-2 and other histological features of NAFLD.—Biopsy findings for patients in each group are summarised in Table 2. The NAFLD Activity Score (NAS) increased with increasing fibrosis stage, as did lobular inflammation and ballooning. Within fibrosis groups, PTX-2 levels were not impacted by grade of inflammation, ballooning or steatosis.

Correlation between serum PTX-2 and MRE.—Mean MRE stiffness values significantly increased with increasing fibrosis stage (Table 2), and PTX –2 levels were significantly negatively correlated with MRE values ($r = -0.31$, $P = 0.02$, Figure 3).

DISCUSSION

In this pilot proof of concept study, we report the first measurements of serum PTX-2 levels in patients with chronic liver disease. In this cohort of uniquely well-characterised NAFLD patients, PTX-2 levels were significantly lower in patients with NAFLD and advanced fibrosis compared to patients with early stage disease. Levels below 14.7 µg/mL were strongly predictive of advanced fibrosis. In addition, PTX-2 levels were inversely correlated with increasing fibrosis stage and with MRE liver stiffness values throughout the spectrum of NAFLD-related fibrosis. This decrease in PTX-2 levels is similar to those seen in pulmonary fibrosis patients,¹³ and is unlikely to be due to a generalised decrease in hepatic synthetic capacity given the similar albumin and CRP levels between groups.

Interestingly, PTX-2 levels were also significantly decreased in patients with NAFLD and no fibrosis compared to healthy controls. PTX-2 < 24 µg/mL had a 92% positive predictive value for the diagnosis of NAFLD. Pentraxin-3, a long pentraxin and acute phase reactant that is expressed locally in the setting of an inflammatory stimulus, including in adipose tissues and atherosclerotic lesions, has previously been implicated in obesity and cardiovascular disease.^{30–38} However, PTX-2 is predominantly synthesised in the liver,³⁹ and there are no previously published data on PTX-2 levels in these populations. In this cohort, PTX-2 levels were not significantly correlated with BMI or degree of steatosis on

liver biopsy, though this cohort may not have had sufficient power to demonstrate these relationships. Thus it is uncertain whether hepatic production of PTX-2 may be inhibited by significant lipid accumulation in the liver. Further mechanistic studies will be required to determine the relationship between hepatic steatosis and PTX-2 production.

As PTX-2 is known to play an important role in the inhibition of hepatic stellate cell activation as well as in the regulation of fibrogenesis and wound healing,^{11, 14} repletion with recombinant protein is a promising therapeutic target. The pre-clinical data on administration of exogenous PTX-2 has shown significant benefit in models of several chronic fibrotic diseases, including bleomycin- or TGF-induced lung fibrosis,^{12, 40, 41} ischaemia reperfusion injury,⁷ corneal injury,⁴² and radiation-induced oral mucositis and fibrosis.⁴³ Importantly, pre-clinical testing in models of chronic liver disease including CCl₄- and bile duct ligation-induced liver fibrosis were similarly encouraging.¹⁴ When these liver-injured mice were treated with recombinant PTX-2, fibrosis development was significantly inhibited, as was the activation of fibrogenic myofibroblasts, inflammatory cytokines (IL-1 β , IL-6 and TNF- α), and pro-fibrogenic genes (e.g. collagen- α 1 and α -smooth muscle actin).¹⁴

We acknowledge that there is a relatively small sample size in this study, and that our findings need to be confirmed in a multicenter setting in a larger cohort of patients with biopsy-proven NAFLD. In addition, as PTX-2 levels are also decreased in patients with other chronic fibrotic diseases, such as pulmonary fibrosis, the utility of PTX-2 as a marker of liver fibrosis specifically in these patients may be limited.

Administration of recombinant PTX-2 has now moved into clinical trials. In the first report of its use in healthy volunteers and patients with pulmonary fibrosis, the infusion was well-tolerated and resulted in a decrease in the percentage of circulating fibrocytes,¹³ with larger clinical trials now ongoing.¹⁵ Given hepatic origin of PTX-2 and the significantly altered levels even in patients with early stage disease, NAFLD patients with fibrosis may be an ideal population to study with PTX-2 based interventions.

In summary, this pilot proof of concept study demonstrates that serum PTX-2 levels are significantly lower in patients with NAFLD compared to normal controls, and decline further with advancing fibrosis stage in a dose-dependent manner. This study, along with pre-clinical data on the use of recombinant PTX-2 in animal models of liver fibrosis and safety data from ongoing clinical trials in humans, may provide justification for testing of PTX-2 administration in patients with biopsy-proven NAFLD-related fibrosis.

ACKNOWLEDGEMENTS

Declaration of personal interests: The study sponsor(s) had no role in the study design, collection, analysis, interpretation of the data, and/or drafting of the manuscript. All authors report that no conflicts of interest exist.

Declaration of funding interests: The study was supported by an investigator initiated study mechanism funded by Promedior Inc. The study was conducted at the Clinical and Translational Research Institute, University of California at San Diego. RL was supported in part by the American Gastroenterological Association (AGA) Foundation – Sucampo – ASP Designated Research Award in Geriatric Gastroenterology and by a T. Franklin Williams Scholarship Award; Funding was provided by Atlantic Philanthropies, Inc, the John A. Hartford Foundation, the Association of Specialty Professors and the American Gastroenterological Association (grant K23-DK090303).

Aliment Pharmacol Ther. Author manuscript; available in PMC 2021 March 20.

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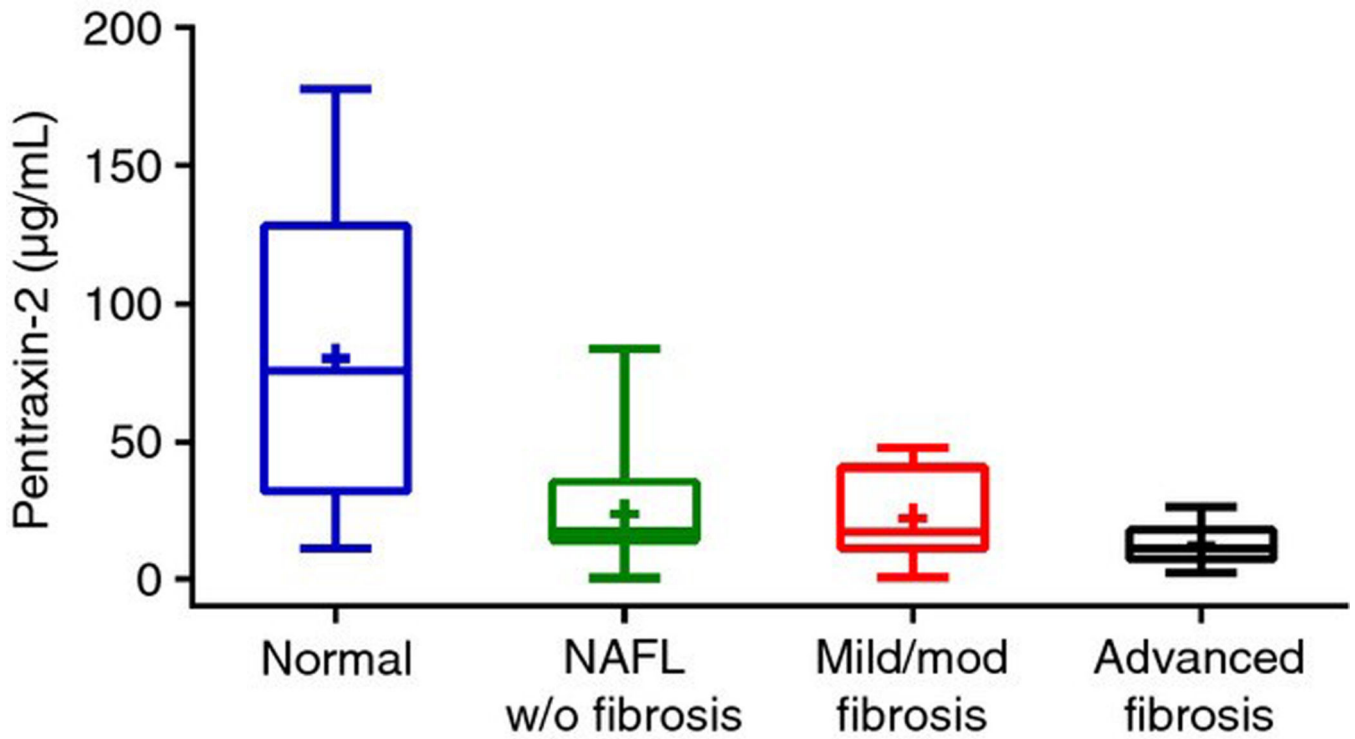


Figure 1 |.

Boxplot of serum PTX-2 levels by patient group. Mean (95% confidence interval) PTX-2 levels were significantly lower in patients with advanced fibrosis [12.2 (−4.3–28.7) µg/mL] compared to those with moderate [22.0 (5.5–38.6) µg/mL] and mild [23.7 (7.2–40.2) µg/mL] NAFLD.

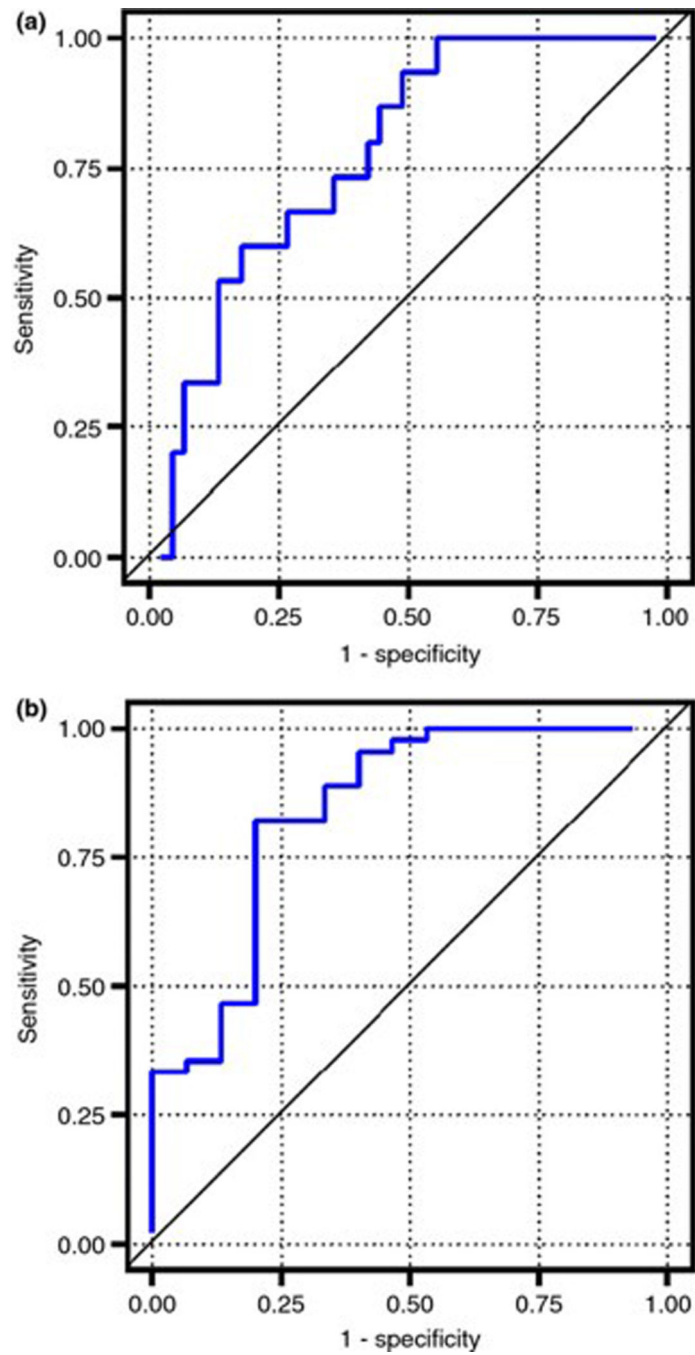


Figure 2 |.

Receiver operating characteristic curves for PTX-2. (a) PTX-2 level had an AUROC of 0.77 (95% CI: 0.65–0.90) for the identification of patients with advanced fibrosis. (b) PTX-2 had an AUROC of 0.84 (95% CI: 0.71–0.97) for the identification of NAFLD compared with normal controls.

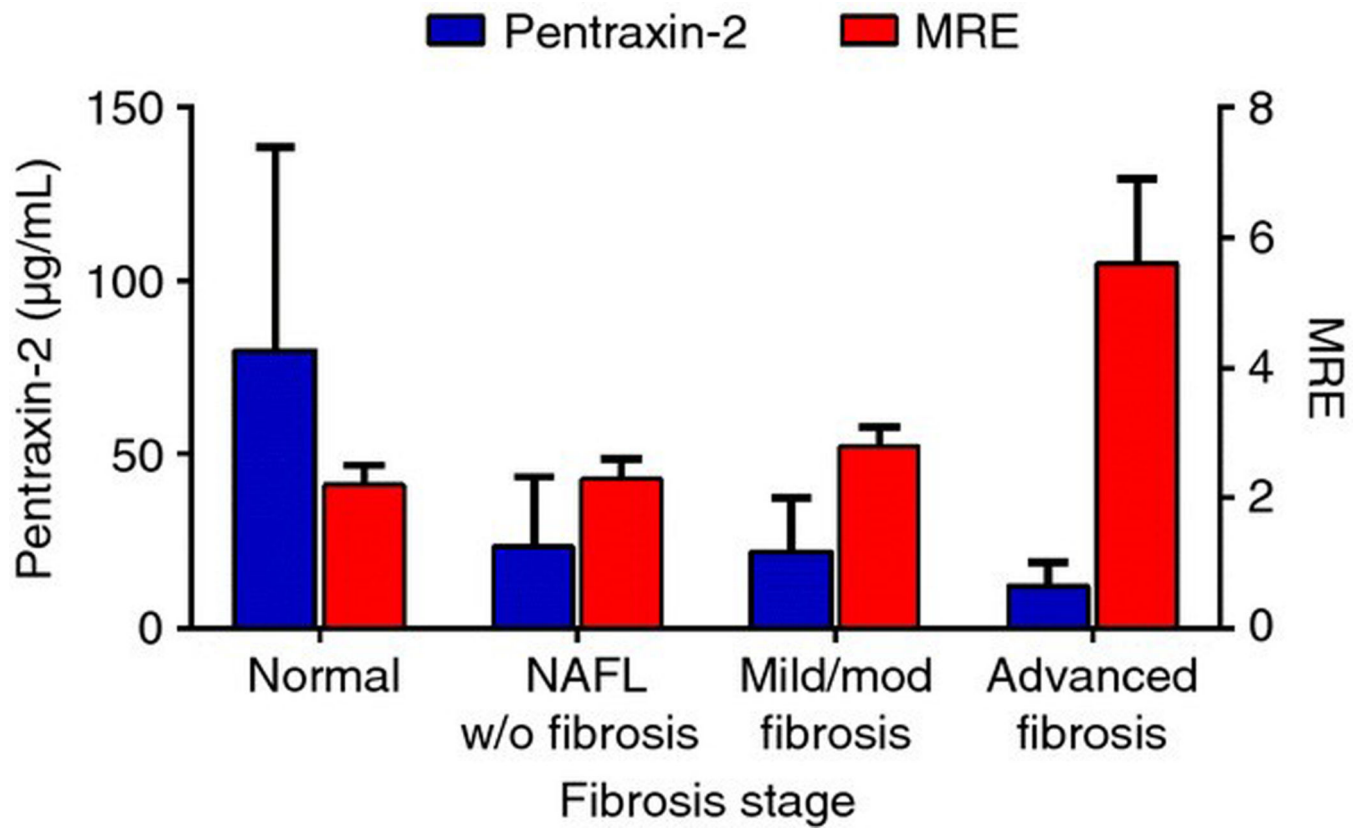


Figure 3 |.
Bar graph demonstrating the negative correlation between PTX-2 and MRE liver stiffness.

Table 1 |

Patient characteristics and Pentraxin-2 levels by patient group

Characteristic	Normal (n = 15)	Mild NAFL (n = 15)	Moderate fibrosis (n = 15)	Advanced fibrosis (n = 15)	P*	P†
Demographics						
Age, mean (s.d.), years	59.2 (5.7)	58.9 (5.2)	56.5 (5.6)	61.1 (4.4)	0.1468	0.5944
Female, n (%)	12 (80.0%)	8 (53.3%)	11 (73.3%)	10 (66.7%)	0.5195	0.7096
Race/ethnicity, n (%)						
White	13 (86.7%)	8 (53.3%)	5 (33.3%)	9 (60.0%)	0.0648	
Black	0	1 (6.7%)	0	0		
Hispanic	2 (13.3%)	3 (20.0%)	6 (40.0%)	6 (40.0%)		
Asian	0	2 (13.3%)	3 (20.0%)	0		
Refused	0	1 (6.7%)	1 (6.7%)	0		
Anthropometric, mean (s.d.)						
Body mass index, kg/m ²	25.6 (5.9)	28.9 (3.1)	32.1 (5.8)	33.4 (4.1)	0.0003	<0.0001
Clinical, n (%)						
Diabetes mellitus	0	3 (20.0%)	8 (53.3%)	7 (46.7%)	0.0020	0.0011
Hypertension	4 (26.7%)	5 (33.3%)	8 (53.3%)	13 (86.7%)	0.0045	0.0005
Hypertriglyceridemia	1 (6.7%)	5 (33.3%)	5 (33.3%)	3 (20.0%)	0.2490	0.4128
Laboratories, mean (s.d.)						
Albumin (g/dL)	4.5 (0.3)	4.6 (0.2)	4.6 (0.2)	4.4 (0.3)	0.1581	0.1552
AST (U/L)	22.3 (6.4)	36.9 (21.7)	47.5 (34.7)	58.2 (25.8)	0.0014	<0.0001
ALT (U/L)	18.9 (7.0)	51.0 (35.4)	73.7 (61.6)	58.0 (32.2)	0.0030	0.0030
Bilirubin, total (mg/dL)	0.4 (0.2)	0.6 (0.3)	0.5 (0.3)	0.7 (0.8)	0.3972	0.1346
Platelet count (1000/mm ³)	257.3 (47.9)	252.6 (53.9)	246.9 (49.4)	182.1 (56.4)	0.0007	0.0004
International normalised ratio						
Fasting serum glucose (mg/dL)	92.7 (10.0)	101.3 (15.4)	112.4 (24.1)	116.1 (36.3)	0.0362	0.0044
Haemoglobin A1c (%)	5.8 (0.3)	5.9 (0.3)	6.2 (0.7)	6.4 (0.6)	0.0041	0.0004
Triglycerides (mg/dL)	102.4 (69.0)	151.1 (72.2)	156.0 (68.9)	179.5 (184.2)	0.2858	0.0687
HDL cholesterol (mg/dL)	66.8 (16.5)	56.4 (14.8)	50.9 (13.5)	45.4 (12.8)	0.0013	0.0001
LDL cholesterol (mg/dL)	108.5 (34.6)	120.1 (36.3)	101.5 (39.8)	93.3 (31.8)	0.2353	0.1330
C-reactive protein (mg/L)	1839.9 (3030.8)	2294.7 (3493.3)	4002.6 (4870.4)	3874.9 (2717.2)	0.2593	0.0670

Characteristic	Normal (n = 15)	Mild NAFL (n = 15)	Moderate fibrosis (n = 15)	Advanced fibrosis (n = 15)	P*	P [†]
Pentraxin-2 (µg/mL), mean (95% CI)						
Unadjusted	80.2 (63.7–96.7)	23.7 (7.2–40.2)	22.0 (5.5–38.6)	12.2 (4.3–28.7)	<0.0001	<0.0001
Age, gender adjusted	85.2 (68.0–102.5)	24.2 (7.9–40.6)	25.3 (8.1–42.5)	15.4 (1.6–32.5)	<0.0001	<0.0001
Age, gender, albumin adjusted	85.2 (67.8–102.6)	24.1 (7.6–40.6)	25.3 (8.0–42.7)	16.0 (1.5–33.5)	<0.0001	<0.0001
Age, gender, CRP adjusted	86.5 (68.9–104.2)	24.9 (8.4–41.4)	24.5 (7.1–41.9)	14.9 (–2.3–32.0)	<0.0001	<0.0001

* Continuous variables were analysed with ANOVA, categorical variables were analysed with Fisher's exact test. The P values in bold are statistically significant.

[†] Test of linear trend.

Table 2 |

Biopsy and MRE characteristics by patient group

	Normal (<i>n</i> = 15)	Mild NAFL (<i>n</i> = 15)	Moderate fibrosis (<i>n</i> = 15)	Advanced fibrosis (<i>n</i> = 15)	<i>P</i> [*]	<i>P</i> [†]
NAFLD Activity Score, (NAS)						
Mean (s.d.)	–	3.6 (1.0)	5.2 (0.9)	5.5 (1.2)	<0.0001	<0.0001
2	–	2 (13.3%)	0	0	0.0059	
3	–	5 (33.3%)	0	0		
4	–	5 (33.3%)	3 (20.0%)	4 (26.7%)		
5	–	3 (20.0%)	7 (46.7%)	4 (26.7%)		
6	–	0	4 (26.7%)	3 (20.0%)		
7	–	0	1 (6.7%)	4 (26.7%)		
Lobular inflammation (score), (no. foci per 200 × field), <i>n</i> (%)						
None (0)	–	0	0	0	0.0004	
<2 foci (1)	–	10 (66.7%)	1 (6.7%)	2 (14.3%)		
2–4 foci (2)	–	5 (33.3%)	14 (93.3%)	10 (71.4%)		
>4 foci (3)	–	0	0	2 (14.3%)		
Steatosis						
Grade, <i>n</i> (%)						
<5% (0)	–	0	1 (6.7%)	0	0.2244	
5–33% (1)	–	9 (60.0%)	3 (20.0%)	6 (40.0%)		
33–66% (2)	–	5 (33.3%)	8 (53.3%)	5 (33.3%)		
>66% (3)	–	1 (6.7%)	3 (20.0%)	4 (26.7%)		
Ballooning (score), <i>n</i> (%)						
None (0)	–	6 (40.0%)	1 (6.7%)	0	0.0010	
Few (1)	–	7 (46.7%)	9 (60.0%)	3 (21.4%)		
Many (2)	–	2 (13.3%)	5 (33.3%)	11 (78.6%)		
MRE, mean (s.d.)	2.2 (0.3)	2.3 (0.3)	2.8 (0.3)	5.6 (1.3)	<0.0001	<0.0001

* Continuous variables were analysed with ANOVA, categorical variables were analysed with Fisher's exact test. The *P*-values in bold are statistically significant.

[†] Test of linear trend.