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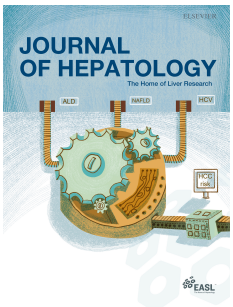

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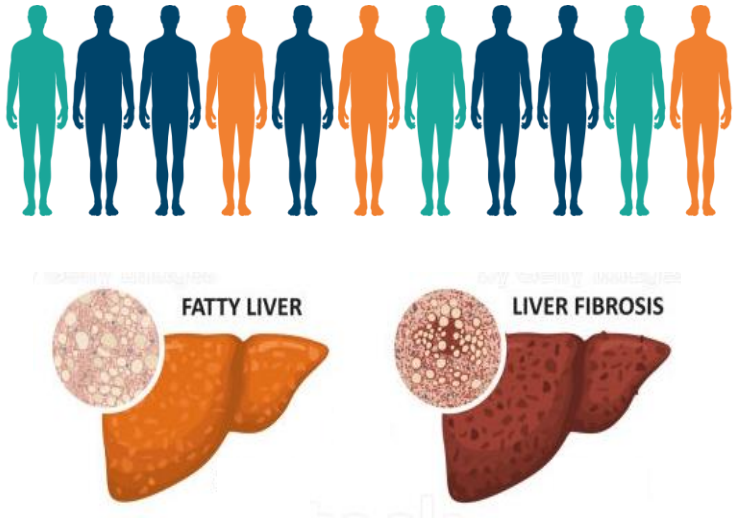
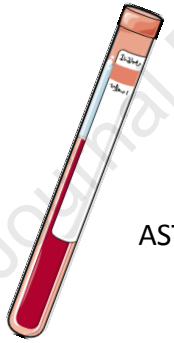
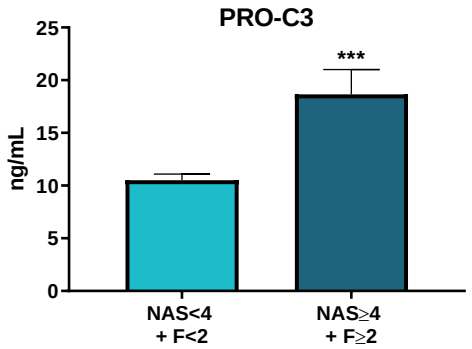
CENTAUR Screening Population
(n=517)





PRO-C3
ADAPT
APRI
FIB4
AST/ALT ratio



	PRO-C3 AUC	ADAPT AUC	APRI AUC	FIB4 AUC	AST/ALT AUC
Significant fibrosis (F2-F4)	0.70	0.76	0.66	0.71	0.58
Advanced fibrosis (F3-F4)	0.73	0.80	0.68	0.79	0.68
NASH	0.74	0.78	0.68	0.68	0.53

Comparison of ADAPT, FIB4 and APRI as non-invasive predictors of liver fibrosis and NASH within the CENTAUR Screening Population

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Author's contributions:

Study concept and design: Pamela Vig, Star Seyedkazemi, Laurent Fischer, Morten Asser Karsdal, Eric Lefebvre, Arun J. Sanyal, Vlad Ratziu

Acquisition of data: Pamela Vig, Star Seyedkazemi, Laurent Fischer, Eric Lefebvre

Analysis and interpretation of data: Mette Juul Nielsen, Diana Julie Leeming, Zachary Goodman, Scott Friedman, Peder Frederiksen, Daniel Kring Rasmussen, Pamela Vig, Star Seyedkazemi, Laurent Fischer, Richard Torstenson, Morten Asser Karsdal, Eric Lefebvre, Arun J. Sanyal, Vlad Ratziu

Statistical analysis: Peder Frederiksen, Mette Juul Nielsen

Drafting of the manuscript: Mette Juul Nielsen and Diana Julie Leeming

Critical revision of the manuscript for important intellectual content: Mette Juul Nielsen, Diana Julie Leeming, Zachary Goodman, Scott Friedman, Peder Frederiksen, Daniel Kring Rasmussen, Pamela Vig, Star Seyedkazemi, Laurent Fischer, Richard Torstenson, Morten Asser Karsdal, Eric Lefebvre, Arun J. Sanyal, Vlad Ratziu

Study supervision: Vlad Ratziu

Clinical trial number: NCT02217475

Data availability statement: Data are available upon request and an appropriate institutional collaboration agreement. Data are not available to access in a repository owing to concern that the identity of patients might be revealed inadvertently.

Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a common liver disorder with non-alcoholic steatohepatitis (NASH) being a more progressive phenotype associated with progression to cirrhosis. Type III collagen is a main component of the fibrotic extracellular matrix. PRO-C3 is a biomarker for detectison of moderate/severe fibrosis and the test is further improved when incorporated into the ADAPT algorithm. Here, we validated PRO-C3 and ADAPT within the CENTAUR screening population.

Methods: PRO-C3 was assessed in plasma from the screening population of the phase IIb CENTAUR study (NCT02217475) in adults with NASH and liver fibrosis. The relation between PRO-C3 and histologic features of NASH was evaluated, as well as the demographics of patients with high and low levels of PRO-C3. The diagnostic ability of PRO-C3 as a stand-alone marker or incorporated into ADAPT to identify patients with F≥2 and NASH was estimated using ROC analysis and logistic regression models.

Results: 517 subjects with matched biopsy and PRO-C3 test were included. Patients with PRO-C3 levels ≥20.2 ng/mL showed increased levels of insulin, HOMA-IR, ALT, AST, alkaline phosphatase, and platelet count compared to patients with low PRO-C3 ($p<0.05$). PRO-C3 increased stepwise with increasing liver fibrosis, lobular inflammation, hepatocyte ballooning, steatosis, and NAS ($p<0.05$), and could separate NAFL from NASH ($p<0.0001$). PRO-C3 was independently associated with fibrosis and NASH when adjusted for clinical confounders. ADAPT outperformed FIB4, APRI, and AST/ALT ratio as predictor of advanced fibrosis and NASH ($p<0.001$).

Conclusion: PRO-C3 was associated with NAS and fibrosis. ADAPT outperformed other non-invasive scores for detecting NASH. These data support the use of PRO-C3 and ADAPT as diagnostic tools to identify patients with NASH eligible for inclusion in clinical trials.

Lay summary: The PRO-C3 is a serological biomarker associated with liver disease activity and fibrosis, and the test is improved when incorporated into the ADAPT score. Here we showed that ADAPT was better at selecting patients with NASH to be included in clinical trials as compared to other non-invasive scores.

INTRODUCTION

The global prevalence of non-alcoholic fatty liver disease (NAFLD) is estimated to be 25% of the population (1). The more severe subtype, non-alcoholic steatohepatitis (NASH), is the progressive form and considered among the top etiologies for hepatocellular carcinoma (HCC) and liver transplantation in the United States. Lack of approved treatment modalities and the growing epidemic of obesity worldwide increase the prevalence of NAFLD, estimated to create a serious health crisis in the next few decades (2). It has recently been recommended by the European Association for the Study of Liver (EASL) to screen high-risk populations for NASH, however, the lack of accurate non-invasive tests (NITs) is hindering effective evaluation of people at risk (3).

Recently, a new definition of fatty liver has been proposed, namely metabolic associated fatty liver disease (MAFLD), as a more appropriate term to describe the liver disease. The diagnosis of MAFLD is based on evidence of hepatic steatosis in addition to one of the following: overweight/obesity, presence of type 2 diabetes, or evidence of metabolic dysfunction regardless of alcohol intake (4).

In the case of chronic liver disease, increased turnover of extracellular matrix (ECM) and an unbalanced formation/degradation process leads to increased liver fibrosis and elevated liver stiffness (5). Collagens are the key players in fibrogenesis and may provide distinct information regarding the nature of fibrosis depending on their localization and function in the ECM (6). Both physiological and pathophysiological relevant proteases are responsible for the generation of ECM protein fragments, so called neo-epitopes, which are found systemically and may be utilized as a biomarker for disease activity when measured in a blood sample (7).

PRO-C3 is a serological biomarker detecting formation of type III collagen, a major scar related collagen that becomes deposited during fibrogenesis by activated myofibroblasts (8). The assay measures the type III collagen formation epitope generated by ADAM-TS2 during release of the N-terminal pro-peptide, in contrast to the classical PIIINP (procollagen III amino terminal propeptide) that measures an internal fragment of PIIINP related to both formation and degradation of type III collagen. PRO-C3 has previously been published as a promising biomarker associated with the degree and change of liver fibrosis (9,10), predict progression of liver fibrosis and outcome (11,12), as well as a pharmacodynamic biomarker in metabolic and biliary liver diseases (13,14). Moreover, PRO-C3 has been incorporated into the composite non-invasive algorithm, ADAPT, which in addition to PRO-C3 includes presence of type 2 diabetes, platelet count, and age (15). ADAPT was able to accurately identify NASH patients with advanced fibrosis superior to other non-invasive algorithms such as FIB4, APRI, and NAFLD fibrosis score (15).

In this study we aimed to investigate the ability of PRO-C3 as a standalone marker and in combination with ADAPT in comparison to standard biomarkers, for the diagnosis of liver fibrosis and correlation to key histological parameters of NAFLD patients within the CENTAUR screening population.

PATIENTS AND METHODS

Study population and design

The study samples originated from the CENTAUR screening population enrolling patients for a phase 2b, randomized, double blind, placebo-controlled, multi-center clinical trial for testing the efficacy and safety of Cenicriviroc (CVC) for the treatment of NASH in adult subjects with liver fibrosis at year 1 and year 2 compared to baseline (NCT02217475) (16). The full description of the CENTAUR study has previously been published (16). Briefly, 812 patients were screened for eligibility for liver biopsy and were in the case of successful biopsy scored according to the NASH Clinical Research Network (CRN) scoring system (17) by one central expert pathologist. Histological evaluation included the NASH CRN fibrosis stage, lobular inflammation grade, hepatocyte ballooning grade, and steatosis grade. The three later grades were combined into the NASH Activity Score (NAS). Stage of fibrosis was also scored according to Ishak scoring system (18). Key inclusion and exclusion criteria for enrollment into the CENTAUR study were as follows:

Key inclusion criteria:

- Males and females; 18–75 years of age
- A biopsy diagnosis of NASH, NAS ≥4, and liver fibrosis stage 1–3
- Documented type 2 diabetes, high body mass index (BMI) >25 kg/m^2 with ≥1 criterion of metabolic syndrome, or bridging fibrosis and/or NAS ≥5
- AST or ALT levels should not exceed x5 normal levels

Key exclusion criteria:

- History of cirrhosis and/or hepatic decompensation including ascites, hepatic encephalopathy, or variceal bleeding
- HBsAg positive, or HCV-Ab positive, with two exceptions if all other eligibility criteria were met.
- Other known causes of chronic liver disease, including alcoholic liver disease, Alcohol consumption (21 units/week for males or 14 units/week for females).
- Pioglitazone, rosiglitazone, vitamin E 400 IU/day, S-adenosylmethionine, pentoxifylline, and ursodiol were disallowed due to possible confounding effect on efficacy

EDTA plasma was collected at the time of biopsy in fasting patients and stored at -80^{o}C for biomarker analysis. A historical biopsy (obtained ≤180 days prior to screening) was used if the subjects were metabolically stable following the procedure and have had no new therapeutic intervention for NASH. Patients without a liver biopsy or an evaluable biopsy were excluded from the analysis. Hematology and serum chemistry laboratory tests were performed by a central laboratory using standard procedures. All patients included in the study provided their written informed consent. Ethics committees in the participating countries have approved the study and the trial has been registered at the National Institutes of Health trial registry (Clinicaltrial.gov, NCT02217475).

Biomarker quantification

Patient plasma samples from the screening population were analyzed in routine biochemical tests using standard methods and assays. Biochemical tests included fasting glucose levels, insulin, HOMA-IR, hemoglobin, HbA1c, white blood cells (WBC), triglycerides, lipoproteins (LDL, HDL, VLDL), total cholesterol, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin, and platelet count. Additional plasma samples were stored at -80°C for future use.

PRO-C3 was analyzed according to manufacturer using a competitive ELISA for the assessment of the formation marker of type III collagen, PRO-C3 (Nordic Bioscience A/S, Herlev, Denmark) as previously described (8). Samples were analyzed according to College of American Pathologists (CAP) guidelines and were blinded to the associated clinical data.

The APRI, AST/ALT ratio, FIB-4, and ADAPT scores were calculated using clinical and routine laboratory variables and previously defined algorithms and cut-off values for NAFLD/NASH patients (19,20).

Statistical Analysis

Summary statistics of the demographics and patients' characteristics were calculated for the overall cohort. Number of subjects, mean, standard deviation (SD), median, and Inter Quartile Range (IQR) were calculated for quantitative variables. For qualitative variables, number of subjects in each category and the frequency were calculated. Comparison between mean marker levels was performed using Kruskal-Wallis test followed by Dunn's multiple comparison test for non-normally distributed variables. The differences between pooled fibrosis stage (F0/1 vs. F2/3/4 and F0/1/2 vs. F3/4) and NAFL/NASH for quantitative variables were compared with Mann-

Whitney test for non-normally distributed variables and p-values were corrected with Bonferroni correction. For qualitative variables, the differences in distribution between pooled fibrosis stages were investigated by Chi-squared test.

Logistic regressions were first used to predict the pooled fibrosis stages. All models were adjusted for age and gender (i.e., these variables were included in the model even if not significant). The models were not adjusted for BMI due to missing values in this screening population for non-enrolled patients, which would lead to fit the models on a subset of the data only. PRO-C3 was used in a univariate logistic regression to assess whether the biomarker could have an impact on the pooled fibrosis stage classification. Relevant clinical variables were first selected with a stepwise multivariable regression. The AIC (Akaike Information Criterion) was used to select variables that improved the model fit (i.e., variables that added information to the model). Then, only the non-correlated variables were kept in the model based on the VIF criterion (Variance Inflation Factor: VIF<2). Only variables with a p-value<0.05 were kept in the model. PRO-C3 was subsequently added to the clinical variables for the pooled fibrosis stages prediction, to adjust the predictive value of PRO-C3 for potential confounders.

State-of-the-art non-invasive scores were calculated to identify patients with advanced fibrosis stages. The scores which could be calculated with variables included in the study were FIB-4, AST/ALT ratio, and APRI, and then compared to PRO-C3 as stand-alone marker or combined with other variables, i.e., the ADAPT score. Predefined cutoff values for those scores were used to evaluate their performance in the CENTAUR screening cohort. The diagnostic accuracy of the different scores was compared using AUROC analyses, and the differences between AUC for the different scores were tested using the Delong test.

The scores were calculated from previously published algorithms:

- APRI (AST to Platelet Ratio Index): $APRI = \frac{\frac{AST\,Level(U/L)}{AST(Upper\,Limit\,of\,Normal)(40U/L)}}{PlateletCount(10^9/L)} \times 100$
- FIB4 (Fibrosis-4): $FIB4 = \frac{Age \times AST(U/L)}{PlateletCount(10^9/L) \times \sqrt{ALT(U/L)}}$
- AST/ALT ratio: $ratio = \frac{AST}{ALT}$
- ADAPT: $ADAPT = \exp(log_{10}(\frac{Age \times PRO-C3(ng/mL)}{\sqrt{PlateletCount(10^9/L)}})) + Diabetes$

Data are shown as median with 95% CI and in graphs as Tukey box and whiskers plot. P-values <0.05 were considered statistically significant. The statistical software R (R-project, Vienna, Austria) or MedCalc Statistical Software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium) were used for all statistical calculations.

RESULTS

Baseline patient characteristics

812 patients were screened for the CENTAUR study of which 607 patients underwent a liver biopsy (**Figure 1**). 570 patients had an evaluable liver biopsy. Sample volume from 44 patients were insufficient for PRO-C3 measurements. Thus, 517 patients with PRO-C3 were included in the present analysis. In this population 52% were male, 41% were diabetic, median age was 55.2, median BMI 32.7, median HbA1c of 6.3%, and within normal ranges of AST, ALT, and ALP levels. BMI, HbA1c, Insulin, and HOMA-IR were only available for 50% of cases corresponding to those enrolled into the CENTUAR study (**Table 1**). All stages of fibrosis were represented (F0-F4) with 29% of patients having a fibrosis stage above F2. 66% of patients had steatohepatitis; 15% of patients had NAS<3, 37% had NAS 3-4, and 48% had NAS>4.

PRO-C3 levels are associated with severity of fibrosis and activity of NASH

Patients were retrospectively stratified according to high or low plasma PRO-C3 by applying a cut-off of 20.2 ng/mL (previous found for detecting fibrosis progressors) (11). Demographics were compared within these two groups (**Table 1**). Patients in the high PRO-C3 group had higher levels of insulin, insulin resistance, ALT, AST, ALP, and lower platelet count compared to patients within the low PRO-C3 group.

When stratifying patients according to fibrosis stage, ballooning, and lobular inflammation, PRO-C3 increased with increasing grades of ballooning ($p<0.001$) (**Figure 2A**), inflammation ($p<0.01$) (**Figure 2B**), steatosis ($p<0.01$) (**Figure 2C**), and with increasing stages of fibrosis ($p<0.001$) (**Figure 2D**). Furthermore, PRO-C3 increased with increasing NAS ($p<0.05$) (**Figure 2E**) and was

notably significant higher in patients with steatohepatitis compared to those with NAFL (p<0.001) (**Figure 2F**). The AUCs for detection of significant fibrosis (F2-4) and advanced fibrosis (F3-4) were 0.71 and 0.73, respectively, p<0.001 for both. The AUC for NASH was 0.74, p<0.001) (**Table 2**). Applying the cut-off of 20.2 ng/mL slightly increased the specificities, but decreased sensitivities, PPVs, and NPVs for the diagnosis of significant and advanced fibrosis, and NASH (**Table 2**).PRO-C3 was then stratified according to both NAS≥4 and fibrosis stage (**Figure 3A**). Here PRO-C3 was significantly elevated in patients with NAS≥4 and fibrosis stage F0, F1, and F3, compared to those with NAS<4 and fibrosis stage F0, F1, and F3 (p<0.05). A trend towards elevated PRO-C3 in patients with NAS≥4 and F2 was observed. When stratifying according to NAS≥4 and fibrosis stage ≥F2, PRO-C3 was significantly elevated in those patients compared to patients with NAS<4 and fibrosis stage <F2 (**Figure 3B**).

In multiple logistic regression analyses, PRO-C3 was independently associated with significant fibrosis (odds ratio (OR)=1.041) when adjusted for gender, age, HDL, bilirubin, AST, hemoglobin, platelets, and WBC. Similar, PRO-C3 was independently associated with advanced fibrosis (OR=1.063) when adjusted for gender, age, presence of diabetes, ALP, platelets, and WBC (**Table 3**).

Validation of ADAPT compared to state-of-the-art biomarkers for liver fibrosis in NAFLD

We compared ADAPT against other NITs including APRI, FIB-4, AST/ALT ratio, and PRO-C3. The choice of algorithms was based on available routine biochemical parameters in the screening data set. APRI, FIB4, and AST/ALT ratio were able to detect significant fibrosis (i.e., F2-F4) with AUCs ranging from 0.58-0.71 ($p<0.01$) (**Table 4**). The diagnostic accuracy of ADAPT was significantly better compared to APRI, FIB4, AST/ALT ratio, and PRO-C3 ($p<0.01$). Likewise, APRI, FIB-4, and AST/ALT ratio were able to detect advanced fibrosis (i.e., F3-F4) with AUCs ranging from 0.68-0.79 ($p<0.001$).

The diagnostic accuracy of ADAPT in significant fibrosis was significantly better compared to APRI, FIB4, AST/ALT ratio, and PRO-C3 ($p<0.001$). In addition, the diagnostic accuracy of ADAPT in presence of advanced fibrosis was significantly better compared to APRI and AST/ALT ratio ($p<0.001$) but performed equally well with FIB4 ($p=0.427$). ADAPT was significantly better to detect NASH as compared to all combinations. APRI and FIB4 were able to detect NASH both with AUCs=0.68 ($p<0.001$). ALT/AST ratio did not significantly detect NASH (AUC=0.53, $p=0.222$).

When investigating the performance of PRO-C3 and ADAPT in predicting significant and advanced fibrosis in two subtypes of MAFLD (i.e., obese and diabetics) similar results were found as compared to the performance in the total population (**Supplementary Fig. 1 and Supplementary Tables 1 and 2**). Unfortunately, the performance of PRO-C3 and ADAPT could not be investigated in the subgroup of non-obese with metabolic dysfunction due to low number of patients in this subgroup (n=9).

In univariate logistic regression analyses, all four non-invasive scores were associated with significant and advanced fibrosis, and NASH (**Table 5**). AST/ALT ratio was however not associated

with NASH in univariate analysis. In multivariable logistic regression analyses, ADAPT was independently associated with significant fibrosis and NASH with ORs of 1.738 and 2.186 ($p<0.001$), respectively. Multivariable logistic regression analysis of advanced fibrosis showed that ADAPT, APRI, and FIB4 were all independently associated (OR 1.883, 0.952, and 3.079, respectively; $p<0.01$).

DISCUSSION

In this study we investigated PRO-C3 and ADAPT as non-invasive diagnostic tools to detect fibrosis and NASH in patients with NAFLD to be enrolled in the CENTAUR phase 2b trial. The main findings of the study were 1) PRO-C3 correlated to disease activity and histological parameters of NASH, 2) PRO-C3 was able to detect patients with significant (F2-F4) and advanced (F3-F4) fibrosis, 3) PRO-C3 was elevated in patients with NASH compared to patients with NAFL, 4) patients with high screening level of PRO-C3 had more insulin resistance and higher liver enzymes compared to patients with low screening level, 5) ADAPT performed better or equally well compared to APRI, FIB4 and AST/ALT ratio for detecting significant and advanced fibrosis, but was superior in detecting and independently associated with NASH compared to APRI, FIB4 and AST/ALT ratio.

There is an unmet clinical need for non-invasive biomarkers that not only accurately can detect the degree of fibrosis, but also identify those patients with high disease activity as the latter group may be more prone to progression of disease and/or optimal responders of a potential therapy (21). Blood tests are attractive alternatives to liver biopsies, as they are easier to perform at a larger scale and are associated with significantly lower procedural risks. However, it may be unlikely that blood tests can provide the same complex information as the liver biopsy, and the blood sample may not reflect a site-specific event as the measured parameter may originate from other sources than the liver. Thus, it is important to identify markers that specifically reflect the ongoing processes of fat accumulation, necroinflammation and fibrogenesis in the liver or the underlying pathophysiologic processes (22).

PRO-C3, a marker of type III collagen synthesis, has been investigated as biomarker related to diagnosis, prognosis, and as pharmacodynamics marker in several studies of patients with liver fibrosis with various underlying etiologies, including viral hepatitis B and C, decompensated alcoholic liver disease, HIV, and NAFLD (9–11,23–27). This study confirms previous findings that PRO-C3 can detect significant or advanced fibrosis and distinguish between patients with and without NASH. Using AUROC analysis to identify the best diagnostic value of a given test provides a cut-off value calculated from the Youden Index corresponding to the point on the curve where the sum of sensitivity and specificity is maximized. In this study, we also used AUROC analysis to detect patients in one group over another resulting in cut-off levels close but not equally to previous cut-off found in other cohorts, however the AUCs are similar. This indicates that there may be cohort specific cut-offs depending on several factors such as underlying etiology, distribution of fibrosis stages, and other confounding factors such as age and metabolic profile. This might explain why we were unable to generate the same cut-off level as we have previously defined in Nielsen et al 2015 (11). However, applying the previously defined cut-off of 20.2 ng/mL in this cohort, showed a worse metabolic profile in patients with high PRO-C3 level compared to those with low PRO-C3 level, indicating that patients with more active disease may be more eligible for inclusion in a clinical trial.

In this study we compared FIB4, APRI, and AST/ALT ratio, which all are indirect NITs of fibrosis, and the newly developed ADAPT to PRO-C3 being direct NITs of fibrosis. We and others have shown that combining several parameters into non-invasive scores, the diagnostic accuracy is improved. A drawback of some of these algorithms are the use of age and presence of diabetes as their performance is expected to be influenced by these parameters as recently reported by McPhearson et al, showing an unacceptable low accuracy of the NAFLD Fibrosis score in patients

aged <35 or >65 (28). When incorporating PRO-C3 in ADAPT, the diagnostic accuracy for detecting fibrosis improved, thus supporting the use of ADAPT for better selection of patients with NAFLD/NASH as previously shown (15). In contrast to APRI and FIB4, ADAPT includes a direct marker of fibrogenesis (PRO-C3) rather than aminotransferases and could therefore be a more accurate measure of hepatic fibrosis. However, using PRO-C3 as a stand-alone biomarker is suboptimal as it measures formation of type III collagen, a protein widely distributed in the body, thus part of the PRO-C3 signal may come from tissues other than the liver. NALFD patients who are older, have diabetes, and low platelet count have a higher risk of having advanced fibrosis, therefore combining disease specific risk factors with a fibrogenesis marker increases the likelihood of correct patient selection. In a recent study by Eslam et al, the diagnostic accuracy was further increased when applying liver stiffness measurements together with ADAPT, thus reducing the requirement for liver biopsy (29). Such approach could be useful in other indications of chronic liver diseases as well, thus allowing for better risk stratification in primary care settings as well as better selection of patients in clinical trials.

In our study, we confirm the use of ADAPT as a diagnostic tool for identifying patients with advanced fibrosis and NASH. In addition, ADAPT was able to outperform APRI, FIB4, and AST/ALT ratio as being independently associated with advanced fibrosis and NASH. Moreover, ADAPT also outperformed APRI, FIB4, and AST/ALT in the subtypes of MALFD, indicating that ADAPT is a reliable biomarker for detecting fibrosis even in subgroups of patients. However, the diagnostic accuracy found in this study is somewhat lower compared to previously published (15). This could be due to differences in patient demographics, such as higher AST, ALT, and platelet count found in the patients reported by Daniels et al, compared to the patients screened for the CENTAUR trial. In addition, in this study PRO-C3 was assessed in EDTA plasma which in general measures

approximately 10% lower compared to serum, which may also influence the cut-off value generated in this study.

Although the samples are from a screening population of a phase 2b trial with full disease spectrum and with liver biopsies scored by a single pathologist, the study has some limitations. While all samples and data collection were obtained prospectively, measurements were done retrospectively. In addition, data on demographics such as BMI, HbA1c, and insulin were not available in almost 50% of subjects as they were classified as screen failures for the phase 2b trial and this information was not captured in the database, making it impossible to calculate the NAFLD fibrosis score from this population and to account for those parameters when investigating the ability of the biomarkers/algorithms to identify significant and advanced fibrosis. Furthermore, data on ELF test was not included in the screening database either, which is a big limitation to the study, as ELF includes the component PIIINP, which also measure type III collagen formation, and the test is therefore the most direct comparator to PRO-C3 and ADAPT. Future analyses include the assessment of PRO-C3 and validation of ADAPT in the CENTAUR phase 2b trial, investigating the ability of PRO-C3 and/or ADAPT as predictors of changes in fibrosis using paired biopsies and as pharmacodynamic markers.

In conclusion we have shown that PRO-C3, a direct NIT of liver fibrosis, is associated with the activity of NASH, and that ADAPT outperformed other indirect non-invasive tests for detecting features of NASH. These data support the use of PRO-C3 and ADAPT as diagnostic tools to identify patients with significant or advanced liver fibrosis associated with NASH who are eligible for inclusion in clinical trials, despite the relatively lower diagnostic accuracy compared to previous reported data.

Reference list:

1. Wong VW, Yilmaz Y, George J, Fan J, Vos MB, Younossi Z, et al. Global Perspectives on Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. 2019;69:2672–2682.
2. Daniels SJ, Leeming DJ, Detlefsen S, Bruun MF, Hjuler ST, Henriksen K, et al. Biochemical and histological characterisation of an experimental rodent model of non-alcoholic steatohepatitis – Effects of a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist and a glucagon-like peptide-1 analogue. Biomed. Pharmacother. 2019;111.
3. Byrne CD, Targher G. EASL–EASD–EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. Diabetologia. 2016;59:1141–1144.
4. Eslam M, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-gomez M, et al. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. J. Hepatol. 2020;73:202–209.
5. Karsdal MA, Manon-Jensen T, Genovese F, Kristensen JH, Nielsen MJ, Sand JMB, et al. Novel insights into the function and dynamics of extracellular matrix in liver fibrosis. Am. J. Physiol. Gastrointest. Liver Physiol. 2015;308:G807-30.
6. Baranova A, Lal P, Birerdinc A, Younossi ZM. Non-invasive markers for hepatic fibrosis. BMC Gastroenterol. 2011;11:91.
7. Karsdal MA, Krarup H, Sand JMB, Christensen PB, Gerstoft J, Leeming DJ, et al. Review article: The efficacy of biomarkers in chronic fibroproliferative diseases - Early diagnosis and prognosis, with liver fibrosis as an exemplar. Aliment. Pharmacol. Ther. 2014;40:233–249.
8. Nielsen MJ, Nedergaard AF, Sun S, Veidal SS, Larsen L, Zheng Q, et al. The neo-epitope specific PRO-C3 ELISA measures true formation of type III collagen associated with liver and muscle parameters. Am. J. Transl. Res. 2013;5:303–315.

9. Luo Y, Oseini A, Gagnon R, Charles ED, Sidik K, Vincent R, et al. An Evaluation of the Collagen Fragments Related to Fibrogenesis and Fibrolysis in Nonalcoholic Steatohepatitis. Sci. Rep. 2018;1–9.

10. Bril F, Leeming DJ, Karsdal MA, Kalavalapalli S, Barb D, Lai J, et al. Use of Plasma Fragments of Propeptides of Type III , V , and VI Procollagen for the Detection of Liver Fibrosis in Type 2 Diabetes. 2019;1–4.

11. Nielsen MJ, Veidal SS, Karsdal MA, Ørsnes-Leeming DJ, Vainer B, Gardner SD, et al. Plasma Pro-C3 (N-terminal type III collagen propeptide) predicts fibrosis progression in patients with chronic hepatitis C. Liver Int. 2015;35:429–437.

12. Nielsen MJ, Thorburn D, Leeming DJ, Hov JR, Nygård S, Moum B, et al. Serological markers of extracellular matrix remodeling predict transplant-free survival in primary sclerosing cholangitis. Aliment. Pharmacol. Ther. 2018;48:179–189.

13. Hirschfield GM, Chazouillères O, Drenth JP, Thorburn D, Harrison SA, Landis CS, et al. Effect of NGM282, a FGF19 analogue, in Primary Sclerosing Cholangitis: a Multicentre, Randomized, Double-Blind, Placebo-Controlled Phase 2 Trial. J. Hepatol. 2019;70:483–493.

14. Harrison SA, Rossi SJ, Paredes AH, Trotter JF, Bashir MR, Guy CD, et al. NGM282 Improves Liver Fibrosis and Histology in 12 Weeks in Patients With Nonalcoholic Steatohepatitis. Hepatology. 2019;1–15.

15. Daniels SJ, Leeming DJ, Eslam M, Hashem AM, Nielsen MJ, Krag A, et al. ADAPT: An algorithm incorporating PRO-C3 accurately identifies patients with NAFLD and advanced fibrosis. Hepatology. 2019;69:1075–1086.

16. Friedman S, Sanyal A, Goodman Z, Lefebvre E, Gottwald M, Fischer L, et al. Efficacy and safety study of cenicriviroc for the treatment of non-alcoholic steatohepatitis in adult

subjects with liver fibrosis: CENTAUR Phase 2b study design. Contemp. Clin. Trials. 2016;47:356–365.

17. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–1321.

18. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. J. Hepatol. 1995;22:696–699.

19. Wai C, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A Simple Noninvasive Index Can Predict Both Significant Fibrosis and Cirrhosis in Patients With Chronic Hepatitis C.

20. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a Simple Noninvasive Index to Predict Significant Fibrosis in Patients With HIV/HCV Coinfection. :1317–1325.

21. Neuschwander-tetri BA. Non-alcoholic fatty liver disease. 2017;1–6.

22. Francque S, Vonghia L. The future of diagnosing NASH - could a simple blood test be the key? Expert Rev. Gastroenterol. Hepatol. [Internet]. 2017;11:995–997. Available from: https://www.tandfonline.com/doi/full/10.1080/17474124.2017.1374851

23. Nielsen MJ, Kazankov K, Leeming DJ, Karsdal MA, Krag A, Barrera F, et al. Markers of collagen remodeling detect clinically significant fibrosis in chronic hepatitis C patients. PLoS One. 2015;10:e0137302.

24. Leeming DJJ, Karsdal MAA, Byrjalsen I, Bendtsen F, Trebicka J, Nielsen MJJ, et al. Novel serological neo-epitope markers of extracellular matrix proteins for the detection of portal hypertension. Aliment. Pharmacol. Ther. 2013;38:1086–1096.

25. Leeming DJ, Anadol E, Schierwagen R, Karsdal MA, Byrjalsen I, Nielsen MJ, et al. Combined antiretroviral therapy attenuates hepatic extracellular matrix remodeling in HIV patients assessed by novel protein fingerprint markers. AIDS. 2014;28.

26. Karsdal MA, Henriksen K, Nielsen MJ, Byrjalsen I, Leeming DJ, Gardner S, et al. Fibrogenesis assessed by serological type III collagen formation identifies patients with progressive liver fibrosis and responders to a potential antifibrotic therapy. Am. J. Physiol. - Gastrointest. Liver Physiol. 2016;311.

27. Nielsen MJMJ, Thorburn D, Leeming DJDJ, Hov JRJR, Nygård S, Moum B, et al. Serological markers of extracellular matrix remodeling predict transplant-free survival in primary sclerosing cholangitis. Aliment. Pharmacol. Ther. 2018;48:179–189.

28. Mcpherson S, Hardy T, Dufour J, Petta S. Age as a Confounding Factor for the Accurate Non-Invasive Diagnosis of Advanced NAFLD Fibrosis. Nat. Publ. Gr. [Internet]. 2016;112:740–751. Available from: http://dx.doi.org/10.1038/ajg.2016.453

29. Eslam M, Wong GL, Hashem AM, Chan HL, Nielsen MJ, Leeming DJ, et al. A Sequential Algorithm Combining ADAPT and Liver Stiffness Can Stage Metabolic-Associated Fatty Liver Disease in Hospital-Based and Primary Care Patients. Am. J. Gastroenterol. 2021;116:984–993.

Figure legends

Figure 1: A Flow chart description of the CENTAUR study screening patients included into the PRO-C3 analysis.

Figure 2: PRO-C3 is related to key histological features of non-alcoholic steatohepatitis (NASH) defined according to the NASH CRN scoring system. PRO-C3 is elevated in more severe A) hepatocellular ballooning grade, B) lobular inflammation grade, C) steatosis grade, D) NASH activity score (NAS), E) Fibrosis stage and F) NAFL versus NASH patients. Data are shown as Tukey box plots. Level of significance *p<0.05; **p<0.001; ***p<0.0001 (A-D: Kruskal-Wallis test; E: Mann-Whitney test).

Figure 3: Plasma PRO-C3 in patients stratified according to A) NASH CRN Fibrosis stage and 4>NAS≥4, and B) NAS≥4 and Fibrosis stage ≥2. Data are shown as Tukey box plots. Level of significance: *p≤0.05; **p≤0.01; ***p<0.001 (Mann-Whitney test).

Tables

Table 1: Patient demographics in the total population and according to PRO-C3 predefined thresholds. [a]517 samples were screened in total; [b]Values were not available in all screened patients. Separation of patients into groups of low PRO-C3 (<20.2 ng/mL) or high PRO-C3 (≥20.2 ng/mL) (26). ALT: alanine aminotransferase; AST: aspartate aminotransferase; HbA1c: hemoglobin A1c; SD: standard deviation, HOMA-IR: Homeostatic model assessment - insulin resistance.

P-values: Mann-Whitney test for continuous variables; Chi-squared test for qualitative variables.

Category	**Total population[a]**		**Low PRO-C3 (<20.2 ng/mL)**		**High PRO-C3 (≥20.2 ng/mL)**		**P value (low vs. high PRO-C3)**
Male, n (%)	517	268 (52)	201 (54)		67 (46)		ns
T2D, n (%)	517	209 (40)	147 (40)		62 (43)		ns
NASH CRN Fibrosis stage, n (%) **F0** **F1** **F2** **F3** **F4**	517	 119 (23) 141 (27) 107 (21) 122 (24) 28 (5)	 112 (30) 114 (31) 75 (20) 62 (17) 9 (2)		 7 (5) 27 (19) 32 (22) 60 (41) 19 (13)		<0.001
NAS score, n (%) **0** **1** **2** **3** **4** **5** **6** **7** **8**	517	 10 (2) 28 (5) 39 (8) 71 (14) 120 (23) 108 (21) 98 (19) 37 (7) 6 (1)	 10 (3) 25 (7) 38 (10) 63 (17) 98 (26) 75 (20) 51 (14) 10 (3) 2 (1)		 0 (0) 3 (2) 1 (1) 8 (6) 22 (15) 33 (23) 47 (32) 27 (19) 4 (3)		<0.001
	n	**Median (95% CI)**	**n**	**Median (95% CI)**	**n**	**Median (95% CI)**	
Age, years	517	55.2 (54.2-56.4)	372	55.2 (53.6-56.5)	145	55.6 (52.5-58.1)	ns
BMI, kg/m^2 [b]	266	32.7 (31.9-33.8)	168	32.3 (31.1-33.5)	98	33.7 (32.3-35.0)	ns

HbA1c, %[b]	265	6.3 (6.0-6.5)	168	6.2 (6.0-6.7)	97	6.3 (6.0-6.6)	ns
Insulin, mIU/L[b]	264	22.4 (20.6-24.3)	167	20.1 (18.2-22.6)	97	26.6 (23.3-30.4)	***0.0009***
HOMA-IR[b]	262	6.7 (5.7-7.2)	165	5.8 (4.9-6.9)	97	7.3 (6.7-8.4)	***0.003***
ALT, U/L	517	47.1 (44.0-50.5)	372	42.0 (38.7-44.3)	145	69.4 (58.6-78.5)	***<0.0001***
AST, U/L	517	34.8 (33.0-37.2)	372	30.7 (29.5-32.2)	145	53.9 (48.7-56.1)	***<0.0001***
Alkaline phosphatase, U/L	517	75.7 (73.5-78.2)	372	74.7 (72.7-77.4)	145	78.6 (74.0-83.8)	***0.03***
Bilirubin, mg/dL	516	0.40 (0.38-0.43)	371	0.39 (0.37-0.41)	145	0.45 (0.40-0.49)	ns
Platelet count, x10^3/mm^3	514	235.0 (227.3-242.0)	372	241 (233-245)	142	221 (207-235)	***0.004***
High density lipoprotein, mg/dL	403	41.0 (40.0-43.0)	282	41.5 (40.0-44.0)	121	41.0 (38.0-43.0)	ns

Table 2: Overview of details and outputs for the PRO-C3 ROC analysis for stratification of patients within F0-1 versus F2-4, F0-2 versus F3-4, or NAFL versus NASH including the area under the curve (AUC), predefined cut-offs for sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) calculations.

Marker	**Pro-C3**		
Stage	F0–1 vs. F2–4	F0–2 vs. F3–4	NAFL vs. NASH
N	260 vs. 257	367 vs. 150	175 vs. 342
Prev %	49.7	29.0	66.2
AUC	0.70	0.73	0.74
95% CI	0.658-0.739	0.687-0.765	0.697-0.775
P value	<0.0001	<0.0001	<0.0001
Cut-off[a]	>19.5	>13.4	>14.7
Sensitivity	44.7	77.3	55.6
Specificity	85.8	59.4	82.3
PPV	75.7	43.8	86.0
NPV	61.1	86.5	48.6
Cut-off[b]	>20.2		
Sensitivity	43.2	52.7	37.4

Specificity	86.9	82.0	90.3
PPV	76.6	54.5	88.3
NPV	60.8	80.9	42.5

a) Cut-off generated from Youden index

b) Predefined cut-off from HCV patients with Ishak 2-4 (11)

Table 3: Overview of details and outputs for PRO-C3 in univariate and multivariable logistic regressions for predicting 1) patients with F0-1 vs. F2-4, 2) patients with F0-2 vs. F3-4, and 3) patients with NAFL vs. NASH.

	Significant fibrosis (F2-F4)		Advanced fibrosis (F3-F4)		NASH	
	P value	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**
Univariate analysis*						
PRO-C3	<0.001	1.071 (1.050-1.095)	<0.001	1.069 (1.050-1.090)	<0.001	1.106 (1.072-1.141)
Multivariable analysis						
PRO-C3	0.005	1.041 (1.013-1.072)	<0.001	1.063 (1.043-1.084)	<0.001	1.092 (1.039-1.148)
Age	<0.001	1.054 (1.031-1.079)	<0.001	1.084 (1.057-1.114)	0.019	1.029 (1.005-1.054)
Gender (M)	0.987	1.005 (0.559-1.812)	0.563	0.869 (0.538-1.401)	0.060	0.580 (0.329-1.024)
ALP	-	-	0.006	1.013 (1.004-1.022)	-	-
AST	0.012	1.019 (1.004-1.034)	-	-	0.008	1.028 (1.007-1.048)
Bilirubin	0.008	3.124 (1.429-7.723)	-	-	-	-
Diabetes (Y)	-	-	<0.001	2.285 (1.449-3.632)	<0.001	3.916 (2.138-7.173)
HDL	<0.001	0.959 (0.940-0.977)	-	-	0.006	0.970 (0.949-0.991)
hemoglobin	0.028	0.790 (0.637-0.973)	-	-	-	-
Platelets	0.026	0.995 (0.991-0.999)	-	-	-	-
WBC	0.002	1.237 (1.085-1.418)	0.033	1.145 (1.011-1.298)	-	-

*) PRO-C3 adjusted for age and gender

Table 4: Overview of details and outputs for ADAPT, APRI, FIB4, AST/ALT ratio and PRO-C3 ROC analyses for stratification of patients within F0-1 versus F2-4, F0-2 versus F3-4, or NAFL versus NASH including the area under the curve (AUC), cut-offs for sensitivity (Sens.%) and specificity (Spec.%), positive predictive value (PPV) and negative predictive value (NPV) calculations. P-values: Delong test.

	Score	AUC (95% CI)	Cut-off	Sens.%	Spec.%	PPV	NPV	p-value compared to ADAPT	p-value compared to PRO-C3
Significant fibrosis (F2-F4)	ADAPT	0.76 (0.72-0.80)	>6.15	0.64	0.75	0.71	0.68	1	<0.001
	APRI	0.66 (0.61-0.71)	>0.42	0.57	0.67	0.63	0.61	<0.001	0.077
	FIB4	0.71 (0.66-0.75)	>1.12	0.71	0.62	0.65	0.69	0.009	0.753
	AST/ALT	0.58 (0.53-0.63)	>0.56	0.90	0.25	0.54	0.71	<0.001	<0.001
	PRO-C3	0.70 (0.66-0.74)	>19.65	0.45	0.86	0.76	0.61	<0.001	1
Advanced fibrosis (F3-F4)	ADAPT	0.80 (0.76-0.84)	>6.16	0.78	0.69	0.50	0.88	1	<0.001
	APRI	0.68 (0.63-0.73)	>0.34	0.79	0.51	0.39	0.86	<0.001	0.048
	FIB4	0.79 (0.75-0.83)	>1.12	0.87	0.59	0.46	0.92	0.479	0.019
	AST/ALT	0.68 (0.64-0.73)	>0.78	0.63	0.64	0.42	0.81	<0.001	0.23
	PRO-C3	0.73 (0.68-0.78)	>13.45	0.77	0.59	0.44	0.87	<0.001	1
NASH	ADAPT	0.78 (0.74-0.82)	>5.50	0.77	0.69	0.83	0.61	1	0.008
	APRI	0.68 (0.63-0.72)	>0.43	0.53	0.75	0.80	0.45	<0.001	0.018
	FIB4	0.67 (0.62-0.72)	>1.12	0.64	0.63	0.77	0.47	<0.001	0.02
	AST/ALT	0.53 (0.48-0.59)	>0.55	0.88	0.25	0.70	0.51	<0.001	<0.001
	PRO-C3	0.74 (0.69-0.78)	>14.75	0.56	0.82	0.86	0.49	0.008	1

Table 5: Overview of details and outputs for ADAPT, APRI, FIB4 and AST/ALT ratio in univariate and multivariable logistic regressions for predicting 1) patients with F0-1 vs. F2-4, 2) patients with F0-2 vs. F3-4, and 3) patients with NAFL vs. NASH.

	Significant fibrosis (F2-F4)		**Advanced fibrosis (F3-F4)**		**NASH**	
	P value	**OR (95%CI)**	**P value**	**OR (95%CI)**	**P value**	**OR (95%CI)**
Univariate analysis						
ADAPT	<0.001	1.829 (1.598-2.095)	<0.001	1.968 (1.707-2.268)	<0.001	2.067 (1.754-2.436)
APRI	<0.001	7.243 (3.435-15.271)	<0.001	4.845 (2.493-9.416)	<0.001	11.511 (4.583-28.907)
FIB4	<0.001	3.283 (2.342-4.602)	<0.001	4.820 (3.34-6.956)	<0.001	2.533 (1.784-3.597)
AST/ALT	0.032	1.918 (1.057-3.479)	<0.001	5.821 (2.969-11.412)	0.744	0.905 (0.497-1.647)
Multivariable analysis						
ADAPT	<0.001	1.696 (1.436-2.003)	<0.001	1.848 (1.539-2.219)	<0.001	2.204 (1.787-2.717)
APRI	-	-	0.002	0.152 (0.047-0.494)	-	-
FIB4	-	-	<0.001	3.180 (0.578-4.526)	-	-
AST/ALT	-	-	-	-	-	-

Figure 1

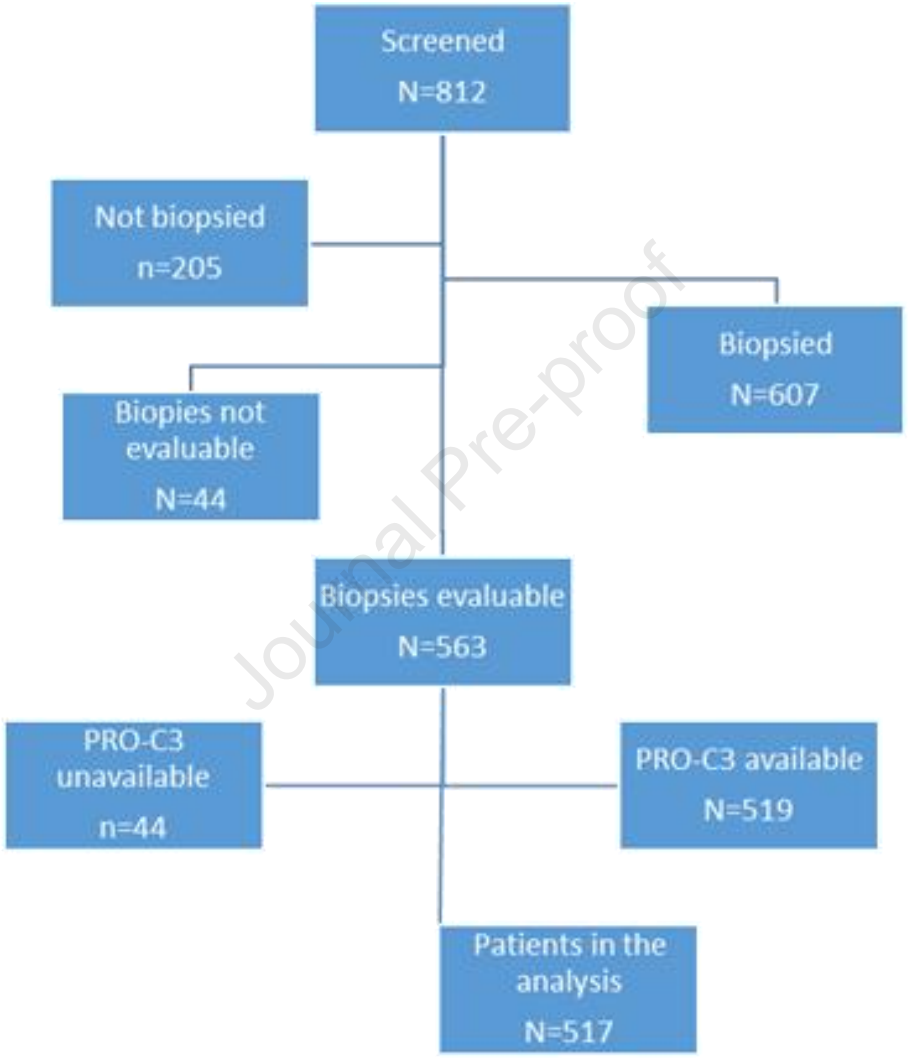


Figure 2

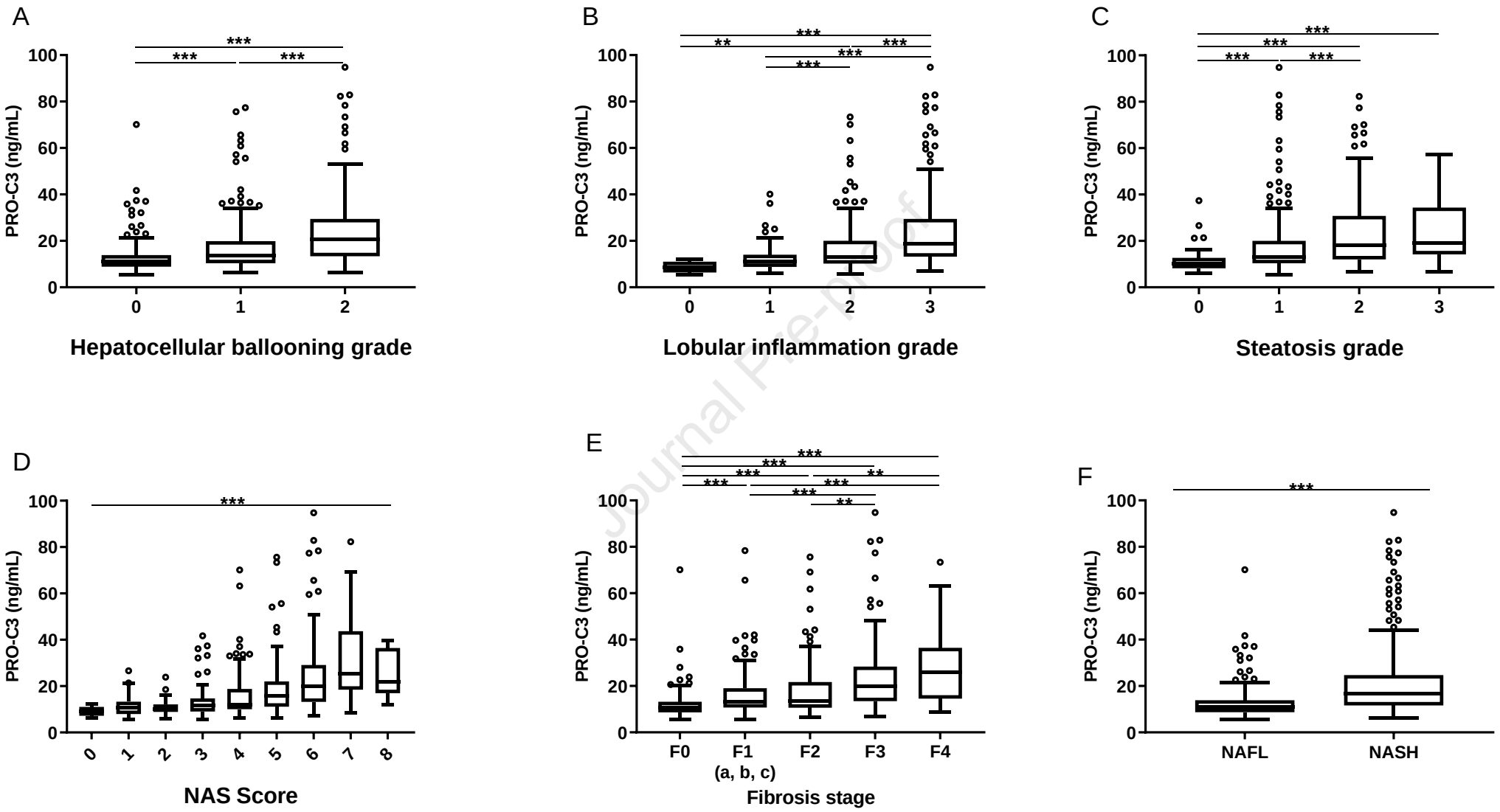
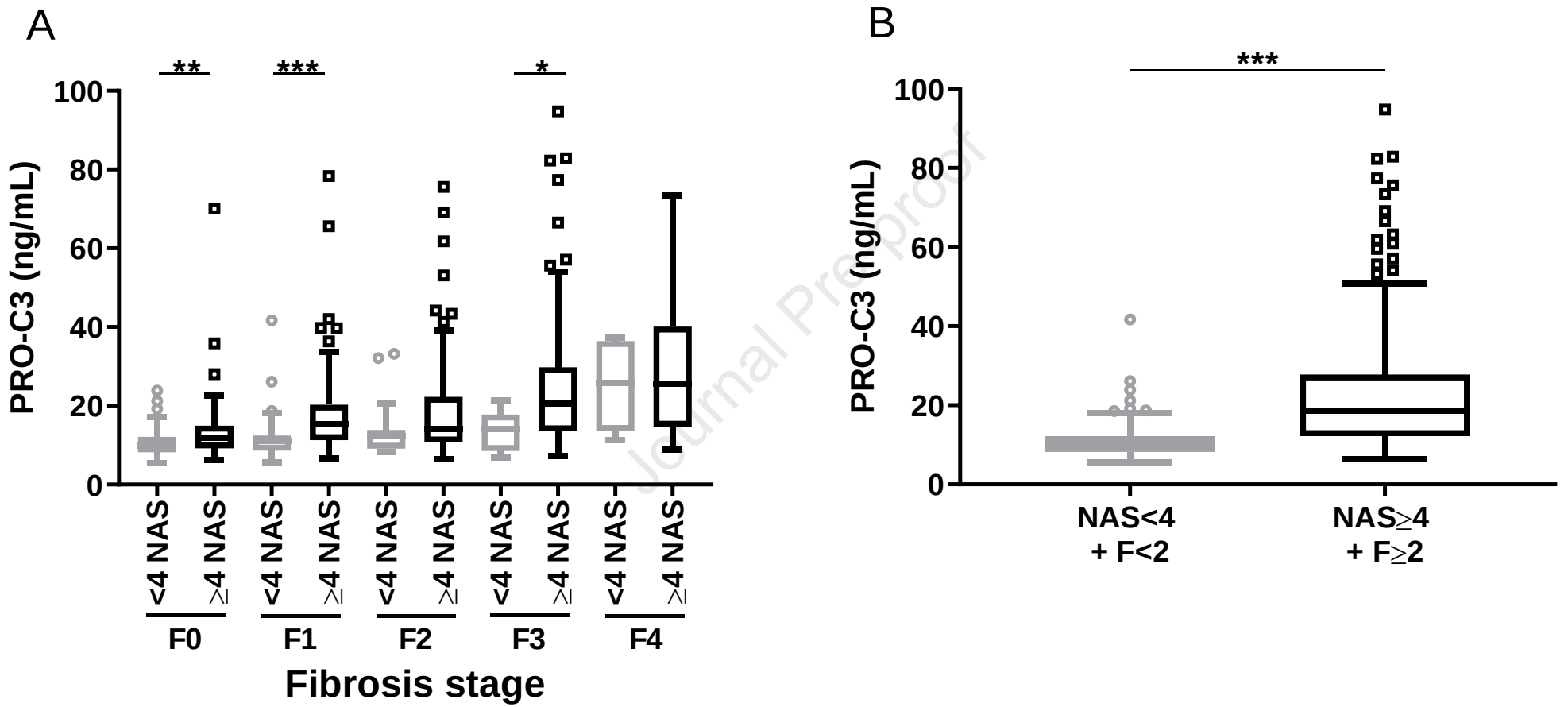


Figure 3



JHEPAT-D-21-01029 Highlights

- It is recommended to screen high-risk populations for NASH; however, accurate non-invasive tests (NITs) are lacking.
- PRO-C3, a direct non-invasive test of liver fibrosis, is associated with the activity of NASH.
- ADAPT, a PRO-C3 based score, outperformed other indirect NITs for detecting features of NASH.
- PRO-C3 and ADAPT are useful tools to identify patients with NASH liver fibrosis eligible for clinical trial inclusion.