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ADAPT: An algorithm incorporating PRO-C3 accurately identifies patients with NAFLD and advanced fibrosis

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List of Abbreviations

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs

ALT: Alanine Aminotransferase

APRI: AST to Platelet Ratio Index

AST: Aspartate Aminotransferase

AUROC: Area under receiver operating curve

BMI: Body mass index

ECM: Extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

GGT: Gamma-Glutamyltransferase

HDL: High-density lipoprotein

LDL: Low-density lipoprotein

LHR+: Positive likelihood ratio

LHR-: Negative likelihood ratio

MS: Metabolic syndrome

NAFL: Non-alcoholic fatty liver

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NFS: NAFLD Fibrosis Score

NPV: Negative predictive value

PPV: Positive predictive value

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Abstract

Background and Aim: Given the high global prevalence of non-alcoholic fatty liver disease (NAFLD), the need for relevant non-invasive biomarkers and algorithms to accurately stage disease severity is a critical unmet medical need. Identifying those with advanced fibrosis (≥F3) is the most crucial, as these individuals have the greatest risk of adverse, long-term, liver-related outcomes. We aimed to investigate the role of PRO-C3 (a marker of type III collagen formation) as a biomarker for advanced fibrosis in NAFLD. Methods: We measured PRO-C3 by enzyme-linked immunosorbent assay (ELISA) in two large independent cohorts with extensive clinical phenotyping and liver biopsy; 150 in the derivation and 281 in the validation cohort. A PRO-C3 based fibrosis algorithm that included Age, presence of DiAbetes, PRO-C3 (a marker of type III collagen formation), and plaTelet count ("ADAPT") was developed. Results: PRO-C3 increased with fibrosis stage (rho 0.50 p<0.0001) and was independently associated with advanced fibrosis (OR=1.05, 95% CI 1.02-1.08, p= 0.003). ADAPT showed areas under the receiver operating characteristics curve (AUROC) of 0.86 (95% CI 0.79 to 0.91) in the derivation and 0.87 in the validation cohort (95% CI 0.83 to 0.91) for advanced fibrosis. This was superior to the existing fibrosis scores, aspartate aminotransferase (AST) to platelet ratio index (APRI), FIB-4 and NAFLD fibrosis score (NFS) in most comparisons. Conclusion: PRO-C3 is an independent predictor of fibrosis stage in NAFLD. A PRO-C3 based score (ADAPT) accurately identifies patients with NAFLD and advanced fibrosis and is superior to APRI, FIB-4 and NFS.

Keywords

Biomarker, Extracellular matrix, Non-invasive score, Non-alcoholic fatty liver disease, PRO-C3

Introduction

The increase in global prevalence of metabolic syndrome (MS) has been accompanied by a rise in organ damage including end stage disease related to non-alcoholic fatty liver disease (NAFLD). Estimates place the worldwide prevalence of NAFLD at 25% [1]. A subset of these patients develop non-alcoholic steatohepatitis (NASH) that can progress to cirrhosis and are at a high risk of adverse liver-related outcomes[1]. From a management and therapeutic perspective, an unmet clinical need is the requirement to distinguish those with early disease from those at highest risk of clinical complications. While metabolic hepatic inflammation is the milieu that drives disease progression, various studies (including meta analyses) that have examined for prognostic histological features suggest that fibrosis stage is the parameter that best associates with overall- and liver-related mortality, as well as liver transplantation and liver related events[2–5].

The gold standard for the evaluation of liver fibrosis stage is percutaneous needle biopsy, which is compromised by inherent sampling and inter-observer biases and peri-procedural risk[6,7]. The invasiveness and costs of performing biopsies also makes it unsuitable for mass screening, for staging and risk stratification. The latter is important as the majority of patients with advanced fibrosis and even cirrhosis, are asymptomatic and often indistinguishable from those at earlier disease stages[8,9]. In this context, there is a need for surrogate markers of disease stage that can identify and risk stratify patients with NAFLD. This area of research can broadly be divided into liquid (typically blood based) or physical approaches (measurement of liver stiffness). Physical approaches while promising are less useful for population level screening and are limited by cost and other technique-specific considerations. Several serum based biomarker tests have previously been developed and applied to NAFLD patients[10–14]. These scores typically combine clinical features and routine laboratory tests and are used primarily to rule out advanced fibrosis[15], however they lack sufficient diagnostic accuracy and sensitivity.

Liver fibrosis is characterized by the accumulation of excess extracellular matrix (ECM) and hence biomarkers reflecting structural changes occurring in the hepatic ECM during chronic injury may be of value in the assessment of fibrosis progression or regression. We recently demonstrated that PRO-C3, an ADAMTS generated neo-epitope marker of type III collagen formation, is a marker of fibrosis in patients with chronic hepatitis C. The role of PRO-C3 in patients with NAFLD however, is largely unknown. Since the performance of biomarkers and non-invasive liver fibrosis scores varies widely according to disease etiology, whether PRO-C3 has a role as a biomarker in NAFLD is unclear. In this study, we sought to a) explore the association of PRO-C3 with liver fibrosis in two large independent biopsy-proven cohorts with NAFLD and b) determine if PRO-C3 can be combined with simple and routinely available clinical variables in to a novel score for the prediction of advanced fibrosis in patients with NAFLD. We compared the performance of our derived model with other known biomarker algorithms.

Materials and Methods

Study population

A total of 431 well phenotyped patients with biopsy confirmed NAFLD comprised the study cohort. The derivation cohort included 150 patients from the Storr Liver Centre, Sydney, Australia; the validation cohort comprised 281 patients recruited from four international sites, Nottingham University Hospitals NHS Trust, United Kingdom (n=42); Kurume University School of Medicine, Kurume, Japan (n=48); University of Western Australia, Nedlands, Australia (n=144) and 47 additional patients from the Storr Liver Centre.

All patients were referred for the investigation of abnormal liver tests or steatosis detected by ultrasound. The diagnosis of NAFLD was established by liver biopsy in all cases. Patients with disease of other etiologies including viral hepatitis and auto-immune liver disease were excluded by standard clinical, laboratory and histopathological assessments. Patients with evidence of hepatic decompensation, secondary causes of steatosis, including excess alcohol (men, >30 g/day; women, >20 g/day), total parenteral nutrition or the use of drugs known to precipitate steatosis were excluded.

Demographic and clinical data were obtained, including age, gender, ethnicity, height, weight, and waist circumference at the time of biopsy. Body mass index (BMI) was calculated as BMI = kg/m^2. Arterial hypertension was defined as blood pressure ≥130/≥85 mmHg or treatment with antihypertensive drugs. Diabetes was defined as a fasting blood glucose ≥7.0 mmol/L, previous diagnosis of diabetes or use of anti-diabetic drugs. Hyperlipidemia was defined as fasting total cholesterol >5.5 mmol/L, triglycerides >1.7 mmol/L or treatment with lipid-lowering drugs. Ethical approval and written informed consent from patients was obtained from all participating centers.

Histology

All biopsies were routinely stained with hematoxylin & eosin and Masson's Trichrome. The stained sections were read and scored by an expert liver pathologist at each participating center using the scoring system proposed by Kleiner *et al*., 2005[16]. The stage of liver fibrosis was defined as: stage 0, absence of fibrosis; stage 1, perisinusoidal or portal fibrosis; stage 2 perisinusoidal and portal/periportal fibrosis; stage 3 septal or bridging fibrosis; and stage 4 as cirrhosis. A diagnosis of NASH was according to the EASL-EASD-EASO guidelines.[17] Thirty-one biopsies were scored independently by pathologists from the various centers, and inter observer agreement was calculated using the κ statistic and was =0.55 for fibrosis, comparable to previously published results[18–21].

Biomarker quantification

At the time of biopsy, a fasting blood sample was obtained and routine biochemical tests were performed using standard methods and assays. Biochemical tests included albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, gamma-glutamyltransferase (GGT), insulin, high-density lipoprotein (HDL), low-density lipoprotein (LDL), platelets, and triglycerides. Additional blood samples were drawn and frozen at −80°C for future research. Type III collagen formation was assessed in serum using the PRO-C3 competitive ELISA assay from Nordic Bioscience, Herlev, Denmark, as previously described[22].

The APRI, FIB-4 and NAFLD Fibrosis Scores were calculated using clinical and routine laboratory variables and previously defined algorithms and cut-off values for NAFLD/NASH patients[10,12,14,23].

Statistical analysis

The main aim of this study was the development of an algorithm comprised of clinical and laboratory variables that could accurately distinguish patients with advanced fibrosis (F≥3) from those without. To this end, patients in the derivation cohort were stratified into those with advanced fibrosis (F≥3) and those without (F0-2). Stage 2 and 3 for lobular and portal inflammation was pooled as only 1 patient in both

groups was graded stage 3. Continuous variables in the two groups were compared using the t test and categorical variables were compared using Fisher's exact test. Comparisons between mean marker levels were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Variables that were significantly different between patients with advanced fibrosis and those without advanced fibrosis were identified as potential algorithm components.

For the formulation of predictive models, variables showing a $p < 0.05$ at univariate analysis (Student t test for parametric variables, and X^2 or Fisher exact test for frequencies) were included. The interaction between these variables was first tested. Variables explaining a statistically significant proportion of the variance ($p < 0.05$) were maintained in the model using the likelihood ratio (LR) test. The model variables were selected using the leave-one-out method to facilitate the calculation of over-fit bias reduced estimates[24]. To avoid over-fitting, 10-fold cross validations were used in the tree building process.

The model was as following:

$$ADAPT = exp\left(log_{10}\left(\frac{Age \times PRO\text{–}C3}{\sqrt{Platelets}}\right)\right) + Diabetes$$

The discriminative ability of the model for the identification of severe fibrosis (F≥3) was assessed by means of receiver operating characteristic curve analysis and expressed as area under the receiver operating characteristic curve (AUROC). A cut-off value to distinguish patients with advanced fibrosis from those without was determined using the bootstrap Youden Index. The diagnostic accuracy of the algorithm and the derived cut-off was determined by calculating sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). To overcome both spectrum effect and ordinal scale issues, we undertook two approaches. Firstly, we used the Obuchowski measure, as proposed by Lambert *et al*[25,26], which is a measure of the probability that two randomly chosen patients from different fibrosis stages are correctly classified according to the weighted scheme, with a penalty for incorrect classification. In the second

method, we standardized the AUROC for the distribution of fibrosis stages as proposed by Poynard *et al*[27], as recently described[28].

ROC curves were also calculated for the established diagnostic scores, APRI, FIB-4 and NAFLD Fibrosis Score. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratio (LHR^+, LHR^-) and 95% CIs were calculated. Estimates of AUROCs and comparisons between AUROCs were performed using the method suggested by Hanley and McNeil[29]. Validation was subsequently performed on the validation cohort as well as for the combined overall cohort.

All data are shown as medians and variation expressed via Tukey plots. P-values <5% were considered significant. Model building and statistical analysis was performed using MedCalc version 16.8.4 (MedCalc Software, Ostend, Belgium) and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Graphs were designed using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA).

Results

Patient Characteristics

The characteristics of the 150 NAFLD patients used to develop the model (derivation cohort) and the 281 used to test the model (validation cohort) are shown in Table 1. Serum levels of albumin, AST, cholesterol and HDL were all significantly lower in the validation cohort when compared to the derivation cohort. In addition, both BMI and insulin level were found to be significantly elevated. No other significant differences were observed between the two cohorts. The prevalence of severe fibrosis was not significantly different between the cohorts.

PRO-C3 is highly associated with severity of fibrosis and histological parameters

Within the derivation cohort, a neo-epitope marker of type III collagen formation, PRO-C3, was found to be significantly elevated in patients with advanced fibrosis (F≥3) compared to the mild/moderate group ($p<0.0001$). PRO-C3 was highly associated with disease severity (Figure 1) and moderately correlated to the severity of fibrosis ($rho = 0.501$, $p<0.0001$). PRO-C3 was able to discriminate between the following stages of fibrosis (Figure 1): F0 versus F2 (27% increase, $p<0.0332$), F0 versus F3 (54% increase, $p<0.0001$), F1 versus F3 (36% increase, $p<0.0002$) and F0 versus F4 (57% increase, $p<0.0021$). In addition, PRO-C3 discriminated between the various stages of hepatocyte ballooning (stage 0 versus stage 1 $p=0.001$, stage 0 versus 2 $p=0.0003$), lobular inflammation (stage 0 versus stage 1 $p=0.0004$, stage 0 versus stage 2 and 3 $p=0.0008$) and steatosis (stage 1 versus stage 3 $p=0.003$).

We undertook logistic regression to discern the effect of various clinical variables on the association of PRO-C3 with the presence of advanced fibrosis (F≥3) within the derivation cohort. In this analysis, PRO-C3, when adjusted for age, ALT, AST, BMI, ballooning, lobular inflammation, presence of diabetes, GGT and platelet count, was independently associated with advanced fibrosis (OR=1.054, 95% CI 1.01-1.07) (Table 2).

The area under the receiver-operating curve (AUROC) for the identification of patients with advanced fibrosis (F≥3) of PRO-C3 alone was 0.81 (95% CI 0.74-0.87) (Data not shown).

Clinical parameters associated with the level of PRO-C3

Given that univariate and multivariate analyses revealed that the level of PRO-C3 was a strong predictor of advanced fibrosis, we examined for clinical parameters associated with the level of PRO-C3. It was subsequently found that ALT (rho 0.29, p=0.0004), AST (rho 0.42, p<0.0001), fasting blood glucose (rho 0.23, p=0.007), insulin level (rho 0.43, p<0.0001), platelet count (rho -0.24, p=0.004) and presence of diabetes (rho 0.16, p=0.05) all correlated with the level of PRO-C3 to varying degrees.

Development of a PRO-C3 based predictive fibrosis score (ADAPT)

Based on the finding that PRO-C3 is strongly associated with fibrosis, we sought to build a model for the prediction of significant fibrosis based on PRO-C3 and routinely assessed clinical and laboratory variables. Patients within the derivation cohort were divided into two groups according to NASH CRN fibrosis stage, F0-2 (no fibrosis to moderate fibrosis) and F3-4 (advanced fibrosis) (Table 3). PRO-C3 was elevated in patients with advanced fibrosis compared to the mild to moderate group (p<0.0001). Furthermore, those with advanced fibrosis had significantly increased levels of AST, GGT and a higher AST/ALT ratio (Table 3). As would be expected, patients with advanced fibrosis had a worse metabolic profile with lower LDL, higher circulating insulin levels and a higher waist-to-hip ratio (Table 3). The presence of diabetes was more likely in patients with advanced fibrosis; 67% of patients with F3-4 had diabetes compared to just 29% of the F0-2 group (p=0.002) (Table 3). In addition, patients with advanced fibrosis were found to be older (p=0.02) and with a lower platelet count compared to those without (p=0.002) (Table 3).

Variables that were significantly different between the two groups ($p<0.05$) were considered eligible for the model building process. Those that described a statistically significant proportion of the variance were included in the model using the likelihood ratio (LR) test. Ultimately, the variables that were included within the model, named "ADAPT", were age, presence of diabetes, platelet count and PRO-C3.

The diagnostic capability of the ADAPT score was assessed via AUROC and was higher than that of PRO-C3 alone, yielding an AUROC of 0.86 (95% CI 0.79-0.91) (Figure 2).

Validation of the diagnostic capabilities of the ADAPT score

To ascertain the validity of our model, the ability of ADAPT to identify patients with advanced fibrosis was corroborated in a separate cohort comprised of patients from four centers across Asia-Pacific and Europe (n=281). Several significant differences were identified between the derivation and the validation cohort; these differences reflect the heterogeneity of NAFLD patients with advanced fibrosis. Despite cohort differences, the diagnostic accuracy of ADAPT was maintained with an AUROC in the validation cohort of 0.87 (95% CI 0.83-0.91) (Figure 3).

The diagnostic performance of a score, when assessed by AUROC, may vary according to disease prevalence, known as spectrum bias[30]. The Obuchowski measure accounts for the spectrum bias and provides a means by which the diagnostic accuracy of a score can be assessed. The Obuchowski measure of ADAPT within the derivation cohort was calculated to be 0.86 and within the validation cohort it was 0.89. Additionally, we standardized the AUROC for the distribution of fibrosis stages according to Poynard *et al*[27]. The standardized AUROC of ADAPT was found to be 0.89 and 0.89 within the derivation and validation cohorts, respectively (Table 4). For further confirmation of the generalizability of the model, the validation cohort was stratified into various groups according to age, (<50, 50-60 and >60), BMI, Sex, NASH vs NAFL and center. In this analysis, ADAPT remained a robust algorithm in that the AUROC was maintained across all sub-populations, with NPV consistently exceeding 90% (Supplementary Figure 1).

Performance of ADAPT against standard algorithms

Within the derivation cohort the AUROC of "ADAPT" (AUROC=0.855) was superior to clinically available serum based non-invasive scores: APRI (AUROC=0.73, p=0.02), FIB-4 (AUROC=0.78, p=0.06) and NAFLD Fibrosis Score (AUROC=0.78, p=0.06) (Table 4). Likewise, in the validation cohort, the AUROC of "ADAPT" (AUROC=0.87) was greater than APRI (AUROC=0.78, p=0.0005), FIB-4 (AUROC=0.85, p=0.32) and NAFLD Fibrosis Score (AUROC= 0.79, p=0.02) (Table 4). Adjusting the AUROC according to Poynard *et al*[27] caused minor increases in the AUROC in all scores (Table 4). Further investigation into the ability of ADAPT to identify patients with clinically significant fibrosis (F2-F4) highlighted the superiority of the ADAPT score when compared to other clinically available serum based non-invasive scores (supplementary table 3).

Derivation of cut-off values

The derivation cohort was subjected to ROC curve analysis to derive a cut-off value for the rule-in and rule-out of advanced fibrosis. A value of >6.3287 for the rule in/out of advanced fibrosis was identified by the Youden Index, PPV 48.4%, NPV 96.6%, (Supplementary table 2). By applying this cut-off, 73% (n=158) F0-2 patients were correctly classified and 27% (n=58) incorrectly classified. Among F3-4 patients, 92% (n=60) were correctly classified while 8% (n=5) were incorrectly classified (Table 5). We applied previously derived cut-off values for APRI (rule in advanced fibrosis >1.5, rule out advanced fibrosis <0.5), FIB-4 (rule in advanced fibrosis >2.67, rule out advanced fibrosis <1.3) and NAFLD Fibrosis Score (rule in advanced fibrosis >0.676, rule out advanced fibrosis <-1.455)[10,14,23]. A large proportion of patients fell within an indeterminate zone, table 5. FIB-4 and APRI showed reasonable performance at identifying patients without advanced liver fibrosis, 68% (n=147) and 67% (n=145) were correctly classified, respectively. However, these scores performed poorly at identifying patients with advanced liver fibrosis, NAFLD Fibrosis Score and FIB-4 correctly identified 51% (n=33) and 46% (n=30) patients, respectively.

Discussion

In this study, we measured PRO-C3 in NAFLD patients from centers across the world and with a wide variation in ages and clinical manifestations, similar to that observed in daily clinical practice. The principal findings were that: 1) PRO-C3 progressively increases with fibrosis severity in NAFLD but that the association remains highly significant even after adjustment for multiple biochemical and clinical parameters, and 2) PRO-C3 when combined with routine clinical parameters (ADAPT) generated a highly accurate tool for the detection of advanced fibrosis in NAFLD. ADAPT is thus a unique score that has utility for risk stratification and for the clinical management of patients with non-alcoholic fatty liver disease.

Non-invasive biomarkers that reflect the process of hepatic fibrosis are urgently needed; collagen formation biomarkers are thus attractive targets. Here we demonstrate that PRO-C3, which measures type III collagen synthesis is a novel and precise marker for advanced liver fibrosis in concordance with what we have recently shown in chronic hepatitis C[22,31,32]. Notably, a recent small non-biopsy study (n=297) from a phase III study of balaglitazone in patients with late-stage Type 2 diabetes (BALLET study) suggested that PRO-C3 could have utility as a determinant of treatment response to a potential anti-fibrotic therapy[33]. Karsdal et al (2016) subsequently confirmed this within a study investigating the anti-fibrotic efficacy of farglitazar[33]; Harrison et al (2018) further explored PRO-C3 as a determinant of treatment response within a phase IIb study[34]. Though that finding needs to be validated in biopsy proven cohorts, their data in combination with our findings suggest that PRO-C3 could serve as a biomarker not only for prediction of fibrosis progression, but also for treatment response. Interestingly, the optimal cut off value in our study was 15.6 ng/ml for advanced fibrosis, which is significantly different from that in patients with hepatitis C (20 ng/ml)[35]. Consistently, the cut off level for PRO-C3 was also lower in the BALLET report (13.1 ng/ml)[33]. Further studies will be required to confirm the optimal cut off in NAFLD. It is noteworthy that the levels of PRO-C3 did not increase from F3 to F4. The explanation for this finding is not clear and further mechanistic studies are required.

Previous reports have suggested that the pro-peptide of type III collagen[22,36] can be used as a biomarker for NASH. However, we have shown that PRO-C3 is distinct from PIIINP in that it is a true marker of type III collagen formation and by extension, fibrogenesis[22]. We subsequently developed a novel PRO-C3-based fibrosis score for NAFLD patients and compared it to various composite serum based score systems that have been proposed and tested in NAFLD patients, namely APRI, FIB-4 and the NAFLD Fibrosis Score. The AUROCs for the various scores examined in this study, all performed similar to previous reports for the identification of advanced fibrosis[27,37–39]. In contrast, ADAPT was superior, as also in the multi-national validation cohort. Critically, ADAPT was robust at identifying patients with advanced fibrosis across different subpopulations (diabetics vs non-diabetics, NAFL vs NASH, various age ranges and BMI categories), some of which have been shown to confound non-invasive algorithms[40,41]. The AUROC of ADAPT was maintained at >0.80 for all subpopulations, while the PPV and NPV remained consistent. From a management perspective, after the application of a derived cut-off value, ADAPT correctly classified 74% of patients without advanced fibrosis and 92% with advanced fibrosis. Cut-off values for APRI, FIB-4 and NAFLD Fibrosis Score were applied to our patients; similar to previous reports, we found that a large proportion of patients fell within an indeterminate zone [37]. FIB-4 and APRI showed reasonable performance at identifying patients without advanced fibrosis, but performed poorly at identifying patients with advanced fibrosis. Furthermore, the superiority of ADAPT is exemplified by its robust performance across various sub-populations (supplementary table 1) and by the substantially higher NPV. In contrast, the performance of FIB-4 has been demonstrated to be variable and is affected by confounders such as age. Additionally, unlike FIB-4, ADAPT is unburdened by the presence of an intermediate zone, which hinders its accuracy[37,41–44]. An advantage of PRO-C3 used alone or in combination as in ADAPT, is that it may stratify cirrhosis since the score is on a spectrum. This contrasts with FIB-4 or the NAFLD fibrosis score which are based on a dichotomous threshold. Hence, PRO-C3 based scores may have potential in patient monitoring over time, though this needs validation.

In contrast to the other non-invasive scores, ADAPT is distinct in that it combines PRO-C3 with important clinical and metabolic parameters associated with disease severity. Both increased age and the presence of diabetes are well-established risk factors for progressive liver disease and are easily discerned[45]. Similarly, platelet count is routinely measured and is strongly correlated with liver fibrosis and has been incorporated into multiple other non-invasive scoring systems[10,12,14]. A study by Mofrad *et al* has shown that the full spectrum of liver fibrosis stages can be found in patients presenting with liver enzymes in the normal range[8]. In addition, liver enzymes are sensitive to age leading to false positive results. Thus, previous analysis has shown that FIB-4 (and likely also APRI and the NAFLD fibrosis score) cannot be universally applied without modification to all patient groups[41]. The lack of inclusion of liver enzymes in ADAPT is thus a conspicuous advantage.

Non-invasive tests have been proposed as screening tools for detecting advanced liver fibrosis in the general population, where the prevalence of this outcome is low[46]. Score systems such as ADAPT, that exhibit a high specificity and NPV could provide a useful tool for clinicians as they reduce any uncertainty surrounding the diagnosis and the number of follow-up assessments required[46]. We propose that the ADAPT score could be used as such a screening tool within the general population to identify patients at risk of or with advanced fibrosis, such that interventions could be applied and progression to cirrhosis perhaps mitigated. However, further validation in non-referral cohorts and demonstration of the cost-effectiveness of using PRO-C3 based score systems is first required.

Our study has some limitations that must be acknowledged. We included well-characterized biopsied patients from centers with an interest in studying NAFLD, therefore referral bias cannot be ruled out. Biopsies were read by an independent pathologist at each participating center using a well-defined and standardized score system. In our hands, the kappa value for assessing the severity of fibrosis has previously been shown to be good[47]. As previously described by Ratziu *et al*, liver biopsy as a diagnostic tool has several limitations including sampling bias[6]. However, all non-invasive diagnostic tools for fibrosis

assessment are benchmarked against the biopsy. Thus, the use of an imperfect reference standard may result in underperformance of the accuracy of non-invasive scores. Additionally, due to the nature of this cross-sectional study, we could not follow the clinical progress of patients; it would be of interest to investigate the relationship of score classification with patient outcome.

In conclusion, a biomarker score based on PRO-C3 and clinical variables (ADAPT) accurately predicts the presence or absence of advanced fibrosis in a NAFLD population. Thus, ADAPT could be useful for risk stratification and management. Further independent studies will be required to determine whether patients stratification using ADAPT followed by measurement of liver stiffness can replace the need for liver biopsy as a diagnostic standard in NAFLD.

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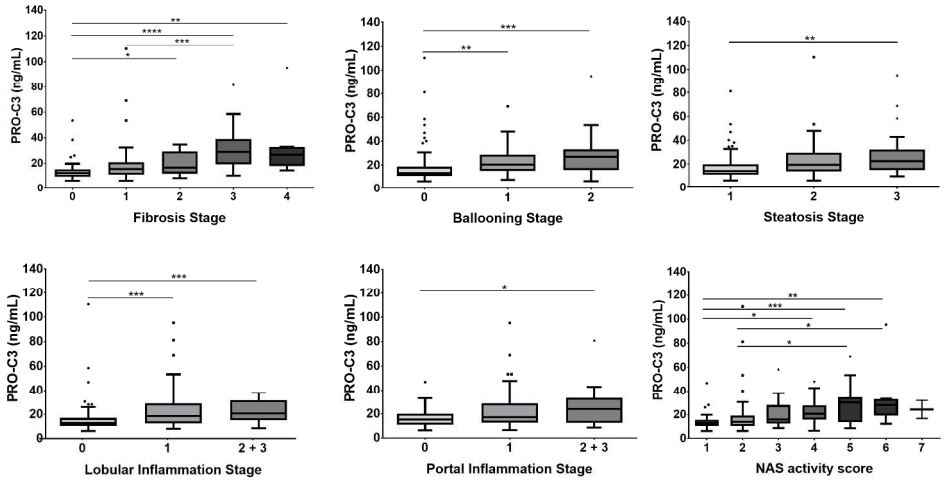

Figure 1: PRO-C3 is highly related to histological parameters of NAFLD‼ ┼ Histological staging according to Kleiner et al 16. Fibrosis stage (rho=0.50, p<0.0001), Ballooning stage (rho=0.38, p<0.0001), Steatosis stage (rho=0.29, p=0.0003), Lobular inflammation (rho=0.36, p<0.0001), Portal inflammation (rho=0.25, p=0.003), NAS activity score (rho=0.46, p<0.0001). * p < 0.0332, ** p < 0.0021, *** p < 0.0002, **** p < 0.0001)

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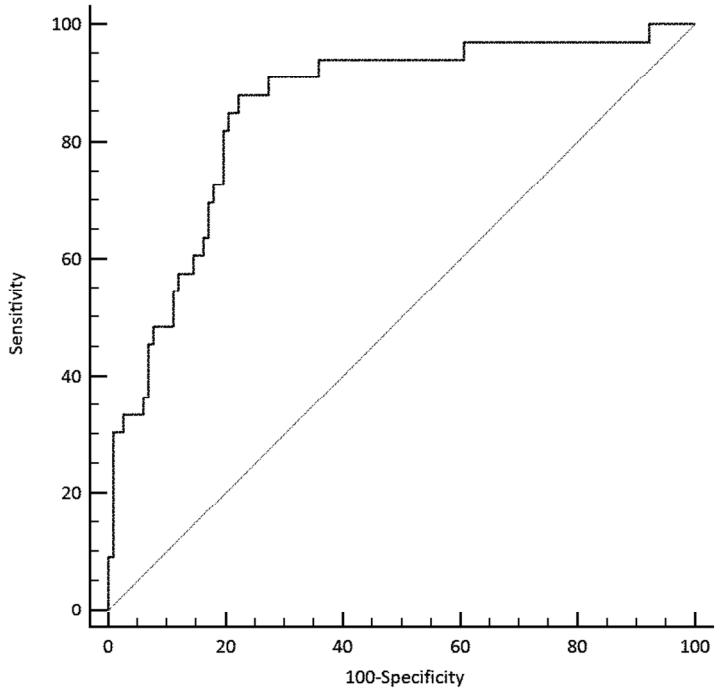

Figure 2: Receiver operating curve (ROC) for the identification of advanced fibrosis by ADAPT within the derivation cohort. AUROC = 0.86 (95% CI 0.79 to 0.91).

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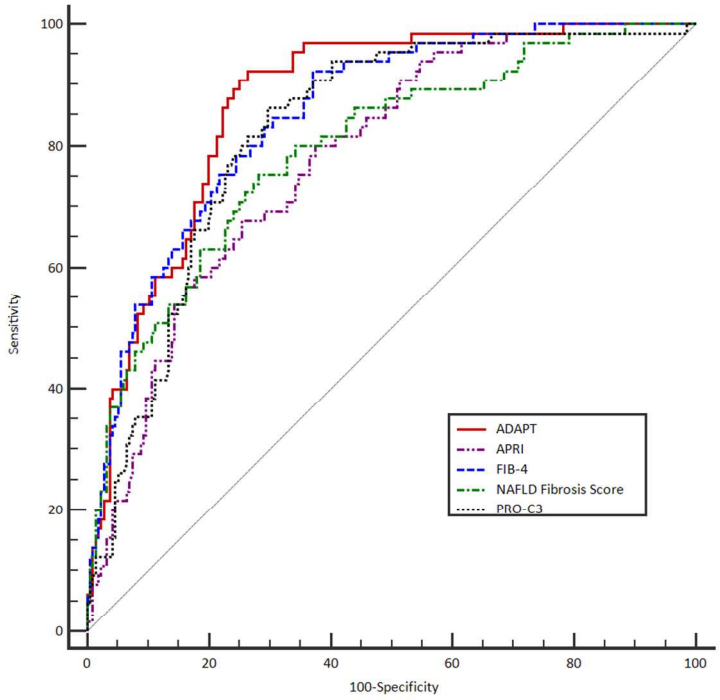

Figure 3: Comparison of various non-invasive serum based scores for the detection of advanced fibrosis (F≥3).

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Table 1. Characteristics of the total patient population

	Derivation Cohort			Validation Cohort			Derivation cohort vs Validation cohort
	n	**Mean**	**SD**	**n**	**Mean**	**SD**	**P-value**
Age (Years)	150	50.85	12.13	277	52.9	12.38	ns
Albumin (g/dL)	148	4.403	5.54	277	4.07	0.40	<0.0001
ALT (IU/L)	148	77.01	50.48	278	69.58	58.43	ns
AST (IU/L)	149	55.02	35.42	262	46.45	33.40	0.02
AST/ALT	148	0.79	0.34	220	0.76	0.38	ns
BMI (kg/m^2)	145	31.3	5.38	274	34.98	9.54	<0.0001
Cholesterol (mmol/L)	148	5.21	1.21	198	4.7	1.15	0.0001
Diabetic	150	37.3%		281	37.4%		ns
Insulin (mIU/L)	147	17.46	12.7	148	26.37	31.81	0.002
FBS (mmol/L)	145	6.46	3.10	239	6.54	2.72	ns
Fibrosis Score (0/1/2/3/4)	42/48/27/25/8			90/87/37/44/21			ns
Gender (% Female)	150	50.7		281	58		ns
GGT (IU/L)	148	128	141	256	112.57	160.24	ns
HDL (mmol/L)	143	1.26	0.41	212	1.2	0.35	0.03
LDL (mmol/L)	140	3.07	0.99	179	2.78	1.56	ns
NASH	55			127			ns

Platelets (×10^9/L)	148	244.4	73.53	270	229.7	79.49	ns
PRO-C3 (ng/mL)	150	20.92	15.48	279	19.93	18.04	ns
Triglycerides (mmol/L)	149	2.03	1.60	263	1.97	1.40	ns
Waist/Hip ratio	136	0.97	0.08	41	0.97	0.09	ns

Table 2 Multivariate logistic regression analyses

	Presence of Advanced Fibrosis	
PRO-C3 Adjusted for	OR (95% CI)	P-value
Unadjusted	1.06 (1.03 to 1.09)	0.0003
Age	1.06 (1.03 to 1.09)	0.0003
ALT	1.07 (1.03 to 1.10)	0.0008
AST	1.05 (1.02 to 1.09)	0.0032
Ballooning	1.05 (1.02 to 1.08)	0.0015
BMI	1.06 (1.02 to 1.09)	0.0004
Diabetes	1.06 (1.03 to 1.09)	0.0002
Gender	1.06 (1.03 to 1.09)	0.0003
GGT	1.05 (1.01 to 1.08)	0.0044
Lobular inflammation	1.05 (1.02 to 1.08)	0.0008
Platelets	1.05 (1.02 to 1.09)	0.0019
Fully Adjusted	1.04 (1.01 to 1.07)	0.0078

Unadjusted and adjusted odds ratios (OR) with 95% confidence intervals (CI) for the increase in PRO-C3

Table 3 Predictors of Advanced Fibrosis

	F=0-2			F=3-4			
	n	**Mean**	**SD**	**n**	**Mean**	**SD**	**P** [a]
Age (Years)	117	49.60	12.28	33	55.30	10.60	0.02
ALT (IU/L)	116	74.31	45.10	32	86.81	66.44	ns
AST (IU/L)	117	51.92	33.30	32	66.34	40.87	0.04
AST/ALT Ratio	116	0.76	0.31	32	0.91	0.39	0.03
BMI (kg/m^2)	113	30.99	5.46	32	32.37	4.98	ns
Diabetes	29%			67%			0.0002[b]
FBSL (mmol/L)	114	6.49	3.38	31	6.37	1.70	ns
GGT (IU/L)	116	111.53	124.14	32	187.78	180.08	0.006
HDL (mmol/L)	114	1.26	0.38	29	1.29	0.49	ns
Insulin (mIU/L)	115	15.37	10.58	31	25.74	16.25	<0.0001
LDL (mmol/L)	112	3.19	0.96	28	2.57	0.96	0.002
Platelets (×10^9/L)	115	254.17	66.14	33	210.45	87.89	0.002
PRO-C3 (ng/mL)	117	17.87	13.10	33	31.72	18.42	<0.0001
TG (mmol/L)	117	2.03	1.61	32	2.03	1.60	ns
Total cholesterol (mmol/l)	116	5.29	1.12	32	4.89	1.46	ns
Waist\Hip ratio	106	0.96	0.08	30	1.01	0.06	0.001

Univariate analysis of variables to identify potential predictors of advanced fibrosis. [a] T-test was assessed to test for significant differences within continuous variables and [b] Fisher's exact test was used for categorical variables

Table 4

	Derivation Cohort				Validation Cohort			
Non-invasive test	**AUROC**	**AdjAUROC**	**SD**	**95% CI**	**AUROC**	**AdjAUROC**	**SD**	**95% CI**
APRI	0.73	0.76	0.05	0.65 to 0.80	0.78	0.80	0.03	0.73 to 0.83
FIB-4	0.78	0.81	0.05	0.70 to 0.84	0.85	0.87	0.02	0.80 to 0.89
NAFLD Fibrosis Score	0.78	0.82	0.05	0.71 to 0.85	0.79	0.81	0.03	0.74 to 0.84
PRO-C3	0.81	0.85	0.04	0.74 to 0.87	0.83	0.84	0.03	0.78 to 0.87
ADAPT	0.86	0.89	0.04	0.79 to 0.91	0.87	0.89	0.02	0.83 to 0.91

AUROC-area under the receiver operating curve, SD- standard deviation, 95% CI- 95% confidence intervals

AdjAUROC- AUROC that has been adjusted according to the Poynard *et al*

Table 5

	F0-2			F3-4		
	Correctly Identified	**Indeterminate**	**Incorrectly Identified**	**Correctly Identified**	**Indeterminate**	**Incorrectly Identified**
∑		216			65	
APRI	145	63	8	10	36	19
FIB-4	147	54	15	30	25	10
NFS	91	100	25	33	25	7
ADAPT	158	-	58	60	-	5

Number of patients correctly, incorrectly or indeterminately classified by the various non-invasive scores

Supplementary table 1. Performance of ADAPT and derived cut-off value (6.3287) across various sub-populations

	NASH	NAFL	Diabetic	Non-Diabetic	Male	Female	BMI 18.5-24.9	BMI 25-29.9	BMI >30	Age <50	Age 51-60	Age >60	Storr Liver Centre	Western Australia	Japan	UK
N	127	151	104	137	118	163	26	71	183	107	91	83	47	144	48	42
F3-4	49	16	39	15	25	40	6	18	41	7	21	37	12	23	12	18
AUROC	0.81	0.89	0.82	0.86	0.85	0.90	0.87	0.82	0.90	0.88	0.82	0.83	0.79	0.93	0.89	0.75
Sensitivity	93.9	87.5	94.9	80.0	96.0	90.0	100	94.4	90.2	85.7	85.7	97.3	83.3	91.3	100	94.4
Specificity	50.0	86.7	52.3	85.2	65.6	78.9	60	66	78.9	87.0	65.7	54.3	77.1	81.8	55.6	50.0
PPV	54.1	43.8	54.4	39.8	42.9	58.1	42.9	48.6	55.2	31.6	42.9	63.2	55.5	48.8	55.6	58.6
NPV	92.9	98.3	94.5	97.2	98.4	96.0	100	97.2	96.5	98.9	93.9	96.1	93.1	98.0	100	92.2

Supplementary Table 2

Score value	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
>6.3287	**90.91 (75.6-98.0)**	**72.65 (63.6-80.5)**	**48.4 (21.5-76.0)**	**96.6 (77.01-100)**
>7.0538	75.76 (57.7-88.9)	80.34 (72.0-87.1)	52.1 (37.2-66.7)	92.2 (85.1-96.6)
>8.4143	48.48 (30.8-66.5)	92.31 (85.9-96.4)	64 (42.5-82.0)	86.4 (79.1-91.9)
>9.5261	33.33 (18.0-51.8)	97.44 (92.7-99.5)	78.6 (49.2-95.3)	83.8 (76.5-89.6)

PPV-positive predictive value, NPV-negative predictive value, 95% CI- 95% confidence intervals

Supplementary Table 3

Identification of F2-4 within Derivation cohort				
Variable	**AUC**	**SE**	**95% CI**	**Vs ADAPT (P=)**
APRI	0.66	0.05	0.58 to 0.73	0.04
FIB-4	0.68	0.04	0.60 to 0.76	0.04
NAFLD Fibrosis Score	0.66	0.05	0.58 to 0.74	0.01
ADAPT	0.76	0.04	0.69 to 0.83	-
Identification of F2-4 within Validation cohort				
APRI	0.81	0.03	0.75 to 0.85	0.03
FIB-4	0.82	0.03	0.76 to 0.86	0.04
NAFLD Fibrosis Score	0.74	0.03	0.68 to 0.79	< 0.0001
ADAPT	0.86	0.02	0.81 to 0.90	-

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ADAPT: An algorithm incorporating PRO-C3 accurately identifies patients with NAFLD and advanced fibrosis

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List of Abbreviations

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs

ALT: Alanine Aminotransferase

APRI: AST to Platelet Ratio Index

AST: Aspartate Aminotransferase

AUROC: Area under receiver operating curve

BMI: Body mass index

ECM: Extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

GGT: Gamma-Glutamyltransferase

HDL: High-density lipoprotein

LDL: Low-density lipoprotein

LHR+: Positive likelihood ratio

LHR-: Negative likelihood ratio

MS: Metabolic syndrome

NAFL: Non-alcoholic fatty liver

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NFS: NAFLD Fibrosis Score

NPV: Negative predictive value

PPV: Positive predictive value

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Abstract

Background and Aim: Given the high global prevalence of non-alcoholic fatty liver disease (NAFLD), the need for relevant non-invasive biomarkers and algorithms to accurately stage disease severity is a critical unmet medical need. Identifying those with advanced fibrosis (≥F3) is the most crucial, as these individuals have the greatest risk of adverse, long-term, liver-related outcomes. We aimed to investigate the role of PRO-C3 (a marker of type III collagen formation) as a biomarker for advanced fibrosis in NAFLD. Methods: We measured PRO-C3 by enzyme-linked immunosorbent assay (ELISA) in two large independent cohorts with extensive clinical phenotyping and liver biopsy; 150 in the derivation and 281 in the validation cohort. A PRO-C3 based fibrosis algorithm that included Age, presence of DiAbetes, PRO-C3 (a marker of type III collagen formation), and plaTelet count ("ADAPT") was developed. Results: PRO-C3 increased with fibrosis stage (rho 0.50 p<0.0001) and was independently associated with advanced fibrosis (OR=1.05, 95% CI 1.02-1.08, p= 0.003). ADAPT showed areas under the receiver operating characteristics curve (AUROC) of 0.86 (95% CI 0.79 to 0.91) in the derivation and 0.87 in the validation cohort (95% CI 0.83 to 0.91) for advanced fibrosis. This was superior to the existing fibrosis scores, aspartate aminotransferase (AST) to platelet ratio index (APRI), FIB-4 and NAFLD fibrosis score (NFS) in most comparisons. Conclusion: PRO-C3 is an independent predictor of fibrosis stage in NAFLD. A PRO-C3 based score (ADAPT) accurately identifies patients with NAFLD and advanced fibrosis and is superior to APRI, FIB-4 and NFS.

Keywords

Biomarker, Extracellular matrix, Non-invasive score, Non-alcoholic fatty liver disease, PRO-C3

Introduction

The increase in global prevalence of metabolic syndrome (MS) has been accompanied by a rise in organ damage including end stage disease related to non-alcoholic fatty liver disease (NAFLD). Estimates place the worldwide prevalence of NAFLD at 25% [1]. A subset of these patients develop non-alcoholic steatohepatitis (NASH) that can progress to cirrhosis and are at a high risk of adverse liver-related outcomes[1]. From a management and therapeutic perspective, an unmet clinical need is the requirement to distinguish those with early disease from those at highest risk of clinical complications. While metabolic hepatic inflammation is the milieu that drives disease progression, various studies (including meta analyses) that have examined for prognostic histological features suggest that fibrosis stage is the parameter that best associates with overall- and liver-related mortality, as well as liver transplantation and liver related events[2–5].

The gold standard for the evaluation of liver fibrosis stage is percutaneous needle biopsy, which is compromised by inherent sampling and inter-observer biases and peri-procedural risk[6,7]. The invasiveness and costs of performing biopsies also makes it unsuitable for mass screening, for staging and risk stratification. The latter is important as the majority of patients with advanced fibrosis and even cirrhosis, are asymptomatic and often indistinguishable from those at earlier disease stages[8,9]. In this context, there is a need for surrogate markers of disease stage that can identify and risk stratify patients with NAFLD. This area of research can broadly be divided into liquid (typically blood based) or physical approaches (measurement of liver stiffness). Physical approaches while promising are less useful for population level screening and are limited by cost and other technique-specific considerations. Several serum based biomarker tests have previously been developed and applied to NAFLD patients[10–14]. These scores typically combine clinical features and routine laboratory tests and are used primarily to rule out advanced fibrosis[15], however they lack sufficient diagnostic accuracy and sensitivity.

Liver fibrosis is characterized by the accumulation of excess extracellular matrix (ECM) and hence biomarkers reflecting structural changes occurring in the hepatic ECM during chronic injury may be of value in the assessment of fibrosis progression or regression. We recently demonstrated that PRO-C3, an ADAMTS generated neo-epitope marker of type III collagen formation, is a marker of fibrosis in patients with chronic hepatitis C. The role of PRO-C3 in patients with NAFLD however, is largely unknown. Since the performance of biomarkers and non-invasive liver fibrosis scores varies widely according to disease etiology, whether PRO-C3 has a role as a biomarker in NAFLD is unclear. In this study, we sought to a) explore the association of PRO-C3 with liver fibrosis in two large independent biopsy-proven cohorts with NAFLD and b) determine if PRO-C3 can be combined with simple and routinely available clinical variables in to a novel score for the prediction of advanced fibrosis in patients with NAFLD. We compared the performance of our derived model with other known biomarker algorithms.

Materials and Methods

Study population

A total of 431 well phenotyped patients with biopsy confirmed NAFLD comprised the study cohort. The derivation cohort included 150 patients from the Storr Liver Centre, Sydney, Australia; the validation cohort comprised 281 patients recruited from four international sites, Nottingham University Hospitals NHS Trust, United Kingdom (n=42); Kurume University School of Medicine, Kurume, Japan (n=48); University of Western Australia, Nedlands, Australia (n=144) and 47 additional patients from the Storr Liver Centre.

All patients were referred for the investigation of abnormal liver tests or steatosis detected by ultrasound. The diagnosis of NAFLD was established by liver biopsy in all cases. Patients with disease of other etiologies including viral hepatitis and auto-immune liver disease were excluded by standard clinical, laboratory and histopathological assessments. Patients with evidence of hepatic decompensation, secondary causes of steatosis, including excess alcohol (men, >30 g/day; women, >20 g/day), total parenteral nutrition or the use of drugs known to precipitate steatosis were excluded.

Demographic and clinical data were obtained, including age, gender, ethnicity, height, weight, and waist circumference at the time of biopsy. Body mass index (BMI) was calculated as BMI = kg/m^2. Arterial hypertension was defined as blood pressure ≥130/≥85 mmHg or treatment with antihypertensive drugs. Diabetes was defined as a fasting blood glucose ≥7.0 mmol/L, previous diagnosis of diabetes or use of anti-diabetic drugs. Hyperlipidemia was defined as fasting total cholesterol >5.5 mmol/L, triglycerides >1.7 mmol/L or treatment with lipid-lowering drugs. Ethical approval and written informed consent from patients was obtained from all participating centers.

Histology

All biopsies were routinely stained with hematoxylin & eosin and Masson's Trichrome. The stained sections were read and scored by an expert liver pathologist at each participating center using the scoring system proposed by Kleiner *et al*., 2005[16]. The stage of liver fibrosis was defined as: stage 0, absence of fibrosis; stage 1, perisinusoidal or portal fibrosis; stage 2 perisinusoidal and portal/periportal fibrosis; stage 3 septal or bridging fibrosis; and stage 4 as cirrhosis. A diagnosis of NASH was according to the EASL-EASD-EASO guidelines.[17] Thirty-one biopsies were scored independently by pathologists from the various centers, and inter observer agreement was calculated using the κ statistic and was =0.55 for fibrosis, comparable to previously published results[18–21].

Biomarker quantification

At the time of biopsy, a fasting blood sample was obtained and routine biochemical tests were performed using standard methods and assays. Biochemical tests included albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, gamma-glutamyltransferase (GGT), insulin, high-density lipoprotein (HDL), low-density lipoprotein (LDL), platelets, and triglycerides. Additional blood samples were drawn and frozen at −80°C for future research. Type III collagen formation was assessed in serum using the PRO-C3 competitive ELISA assay from Nordic Bioscience, Herlev, Denmark, as previously described[22].

The APRI, FIB-4 and NAFLD Fibrosis Scores were calculated using clinical and routine laboratory variables and previously defined algorithms and cut-off values for NAFLD/NASH patients[10,12,14,23].

Statistical analysis

The main aim of this study was the development of an algorithm comprised of clinical and laboratory variables that could accurately distinguish patients with advanced fibrosis (F≥3) from those without. To this end, patients in the derivation cohort were stratified into those with advanced fibrosis (F≥3) and those without (F0-2). Stage 2 and 3 for lobular and portal inflammation was pooled as only 1 patient in both

groups was graded stage 3. Continuous variables in the two groups were compared using the t test and categorical variables were compared using Fisher's exact test. Comparisons between mean marker levels were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Variables that were significantly different between patients with advanced fibrosis and those without advanced fibrosis were identified as potential algorithm components.

For the formulation of predictive models, variables showing a $p < 0.05$ at univariate analysis (Student t test for parametric variables, and X^2 or Fisher exact test for frequencies) were included. The interaction between these variables was first tested. Variables explaining a statistically significant proportion of the variance ($p < 0.05$) were maintained in the model using the likelihood ratio (LR) test. The model variables were selected using the leave-one-out method to facilitate the calculation of over-fit bias reduced estimates[24]. To avoid over-fitting, 10-fold cross validations were used in the tree building process.

The model was as following:

$$ADAPT = exp\left(log_{10}\left(\frac{Age \times PRO\text{–}C3}{\sqrt{Platelets}}\right)\right) + Diabetes$$

The discriminative ability of the model for the identification of severe fibrosis (F≥3) was assessed by means of receiver operating characteristic curve analysis and expressed as area under the receiver operating characteristic curve (AUROC). A cut-off value to distinguish patients with advanced fibrosis from those without was determined using the bootstrap Youden Index. The diagnostic accuracy of the algorithm and the derived cut-off was determined by calculating sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). To overcome both spectrum effect and ordinal scale issues, we undertook two approaches. Firstly, we used the Obuchowski measure, as proposed by Lambert *et al*[25,26], which is a measure of the probability that two randomly chosen patients from different fibrosis stages are correctly classified according to the weighted scheme, with a penalty for incorrect classification. In the second

method, we standardized the AUROC for the distribution of fibrosis stages as proposed by Poynard *et al*[27], as recently described[28].

ROC curves were also calculated for the established diagnostic scores, APRI, FIB-4 and NAFLD Fibrosis Score. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratio (LHR^+, LHR^-) and 95% CIs were calculated. Estimates of AUROCs and comparisons between AUROCs were performed using the method suggested by Hanley and McNeil[29]. Validation was subsequently performed on the validation cohort as well as for the combined overall cohort.

All data are shown as medians and variation expressed via Tukey plots. P-values <5% were considered significant. Model building and statistical analysis was performed using MedCalc version 16.8.4 (MedCalc Software, Ostend, Belgium) and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Graphs were designed using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA).

Results

Patient Characteristics

The characteristics of the 150 NAFLD patients used to develop the model (derivation cohort) and the 281 used to test the model (validation cohort) are shown in Table 1. Serum levels of albumin, AST, cholesterol and HDL were all significantly lower in the validation cohort when compared to the derivation cohort. In addition, both BMI and insulin level were found to be significantly elevated. No other significant differences were observed between the two cohorts. The prevalence of severe fibrosis was not significantly different between the cohorts.

PRO-C3 is highly associated with severity of fibrosis and histological parameters

Within the derivation cohort, a neo-epitope marker of type III collagen formation, PRO-C3, was found to be significantly elevated in patients with advanced fibrosis (F≥3) compared to the mild/moderate group ($p<0.0001$). PRO-C3 was highly associated with disease severity (Figure 1) and moderately correlated to the severity of fibrosis ($rho = 0.501$, $p<0.0001$). PRO-C3 was able to discriminate between the following stages of fibrosis (Figure 1): F0 versus F2 (27% increase, $p<0.0332$), F0 versus F3 (54% increase, $p<0.0001$), F1 versus F3 (36% increase, $p<0.0002$) and F0 versus F4 (57% increase, $p<0.0021$). In addition, PRO-C3 discriminated between the various stages of hepatocyte ballooning (stage 0 versus stage 1 $p=0.001$, stage 0 versus 2 $p=0.0003$), lobular inflammation (stage 0 versus stage 1 $p=0.0004$, stage 0 versus stage 2 and 3 $p=0.0008$) and steatosis (stage 1 versus stage 3 $p=0.003$).

We undertook logistic regression to discern the effect of various clinical variables on the association of PRO-C3 with the presence of advanced fibrosis (F≥3) within the derivation cohort. In this analysis, PRO-C3, when adjusted for age, ALT, AST, BMI, ballooning, lobular inflammation, presence of diabetes, GGT and platelet count, was independently associated with advanced fibrosis (OR=1.054, 95% CI 1.01-1.07) (Table 2).

The area under the receiver-operating curve (AUROC) for the identification of patients with advanced fibrosis (F≥3) of PRO-C3 alone was 0.81 (95% CI 0.74-0.87) (Data not shown).

Clinical parameters associated with the level of PRO-C3

Given that univariate and multivariate analyses revealed that the level of PRO-C3 was a strong predictor of advanced fibrosis, we examined for clinical parameters associated with the level of PRO-C3. It was subsequently found that ALT (rho 0.29, p=0.0004), AST (rho 0.42, p<0.0001), fasting blood glucose (rho 0.23, p=0.007), insulin level (rho 0.43, p<0.0001), platelet count (rho -0.24, p=0.004) and presence of diabetes (rho 0.16, p=0.05) all correlated with the level of PRO-C3 to varying degrees.

Development of a PRO-C3 based predictive fibrosis score (ADAPT)

Based on the finding that PRO-C3 is strongly associated with fibrosis, we sought to build a model for the prediction of significant fibrosis based on PRO-C3 and routinely assessed clinical and laboratory variables. Patients within the derivation cohort were divided into two groups according to NASH CRN fibrosis stage, F0-2 (no fibrosis to moderate fibrosis) and F3-4 (advanced fibrosis) (Table 3). PRO-C3 was elevated in patients with advanced fibrosis compared to the mild to moderate group (p<0.0001). Furthermore, those with advanced fibrosis had significantly increased levels of AST, GGT and a higher AST/ALT ratio (Table 3). As would be expected, patients with advanced fibrosis had a worse metabolic profile with lower LDL, higher circulating insulin levels and a higher waist-to-hip ratio (Table 3). The presence of diabetes was more likely in patients with advanced fibrosis; 67% of patients with F3-4 had diabetes compared to just 29% of the F0-2 group (p=0.002) (Table 3). In addition, patients with advanced fibrosis were found to be older (p=0.02) and with a lower platelet count compared to those without (p=0.002) (Table 3).

Variables that were significantly different between the two groups (p<0.05) were considered eligible for the model building process. Those that described a statistically significant proportion of the variance were

included in the model using the likelihood ratio (LR) test. Ultimately, the variables that were included within the model, named "ADAPT", were age, presence of diabetes, platelet count and PRO-C3.

The diagnostic capability of the ADAPT score was assessed via AUROC and was higher than that of PRO-C3 alone, yielding an AUROC of 0.86 (95% CI 0.79-0.91) (Figure 2).

Validation of the diagnostic capabilities of the ADAPT score

To ascertain the validity of our model, the ability of ADAPT to identify patients with advanced fibrosis was corroborated in a separate cohort comprised of patients from four centers across Asia-Pacific and Europe (n=281). Several significant differences were identified between the derivation and the validation cohort; these differences reflect the heterogeneity of NAFLD patients with advanced fibrosis. Despite cohort differences, the diagnostic accuracy of ADAPT was maintained with an AUROC in the validation cohort of 0.87 (95% CI 0.83-0.91) (Figure 3).

The diagnostic performance of a score, when assessed by AUROC, may vary according to disease prevalence, known as spectrum bias[30]. The Obuchowski measure accounts for the spectrum bias and provides a means by which the diagnostic accuracy of a score can be assessed. The Obuchowski measure of ADAPT within the derivation cohort was calculated to be 0.86 and within the validation cohort it was 0.89. Additionally, we standardized the AUROC for the distribution of fibrosis stages according to Poynard *et al*[27]. The standardized AUROC of ADAPT was found to be 0.89 and 0.89 within the derivation and validation cohorts, respectively (Table 4). For further confirmation of the generalizability of the model, the validation cohort was stratified into various groups according to age, (<50, 50-60 and >60), BMI, Sex, NASH vs NAFL and center. In this analysis, ADAPT remained a robust algorithm in that the AUROC was maintained across all sub-populations, with NPV consistently exceeding 90% (Supplementary Figure 1).

Performance of ADAPT against standard algorithms

Within the derivation cohort the AUROC of "ADAPT" (AUROC=0.855) was superior to clinically available serum based non-invasive scores: APRI (AUROC=0.73, p=0.02), FIB-4 (AUROC=0.78, p=0.06) and NAFLD Fibrosis Score (AUROC=0.78, p=0.06) (Table 4). Likewise, in the validation cohort, the AUROC of "ADAPT" (AUROC=0.87) was greater than APRI (AUROC=0.78, p=0.0005), FIB-4 (AUROC=0.85, p=0.32) and NAFLD Fibrosis Score (AUROC= 0.79, p=0.02) (Table 4). Adjusting the AUROC according to Poynard *et al*[27] caused minor increases in the AUROC in all scores (Table 4). Further investigation into the ability of ADAPT to identify patients with clinically significant fibrosis (F2-F4) highlighted the superiority of the ADAPT score when compared to other clinically available serum based non-invasive scores (supplementary table 3).

Derivation of cut-off values

The derivation cohort was subjected to ROC curve analysis to derive a cut-off value for the rule-in and rule-out of advanced fibrosis. A value of >6.3287 for the rule in/out of advanced fibrosis was identified by the Youden Index, PPV 48.4%, NPV 96.6%, (Supplementary table 2). By applying this cut-off, 73% (n=158) F0-2 patients were correctly classified and 27% (n=58) incorrectly classified. Among F3-4 patients, 92% (n=60) were correctly classified while 8% (n=5) were incorrectly classified (Table 5). We applied previously derived cut-off values for APRI (rule in advanced fibrosis >1.5, rule out advanced fibrosis <0.5), FIB-4 (rule in advanced fibrosis >2.67, rule out advanced fibrosis <1.3) and NAFLD Fibrosis Score (rule in advanced fibrosis >0.676, rule out advanced fibrosis <-1.455)[10,14,23]. A large proportion of patients fell within an indeterminate zone, table 5. FIB-4 and APRI showed reasonable performance at identifying patients without advanced liver fibrosis, 68% (n=147) and 67% (n=145) were correctly classified, respectively. However, these scores performed poorly at identifying patients with advanced liver fibrosis, NAFLD Fibrosis Score and FIB-4 correctly identified 51% (n=33) and 46% (n=30) patients, respectively.

Discussion

In this study, we measured PRO-C3 in NAFLD patients from centers across the world and with a wide variation in ages and clinical manifestations, similar to that observed in daily clinical practice. The principal findings were that: 1) PRO-C3 progressively increases with fibrosis severity in NAFLD but that the association remains highly significant even after adjustment for multiple biochemical and clinical parameters, and 2) PRO-C3 when combined with routine clinical parameters (ADAPT) generated a highly accurate tool for the detection of advanced fibrosis in NAFLD. ADAPT is thus a unique score that has utility for risk stratification and for the clinical management of patients with non-alcoholic fatty liver disease.

Non-invasive biomarkers that reflect the process of hepatic fibrosis are urgently needed; collagen formation biomarkers are thus attractive targets. Here we demonstrate that PRO-C3, which measures type III collagen synthesis is a novel and precise marker for advanced liver fibrosis in concordance with what we have recently shown in chronic hepatitis C[22,31,32]. Notably, a recent small non-biopsy study (n=297) from a phase III study of balaglitazone in patients with late-stage Type 2 diabetes (BALLET study) suggested that PRO-C3 could have utility as a determinant of treatment response to a potential anti-fibrotic therapy[33]. Karsdal et al (2016) subsequently confirmed this within a study investigating the anti-fibrotic efficacy of farglitazar[33]; Harrison et al (2018) further explored PRO-C3 as a determinant of treatment response within a phase IIb study[34]. Though that finding needs to be validated in biopsy proven cohorts, their data in combination with our findings suggest that PRO-C3 could serve as a biomarker not only for prediction of fibrosis progression, but also for treatment response. Interestingly, the optimal cut off value in our study was 15.6 ng/ml for advanced fibrosis, which is significantly different from that in patients with hepatitis C (20 ng/ml)[35]. Consistently, the cut off level for PRO-C3 was also lower in the BALLET report (13.1 ng/ml)[33]. Further studies will be required to confirm the optimal cut off in NAFLD. It is noteworthy that the levels of PRO-C3 did not increase from F3 to F4. The explanation for this finding is not clear and further mechanistic studies are required.

Previous reports have suggested that the pro-peptide of type III collagen[22,36] can be used as a biomarker for NASH. However, we have shown that PRO-C3 is distinct from PIIINP in that it is a true marker of type III collagen formation and by extension, fibrogenesis[22]. We subsequently developed a novel PRO-C3-based fibrosis score for NAFLD patients and compared it to various composite serum based score systems that have been proposed and tested in NAFLD patients, namely APRI, FIB-4 and the NAFLD Fibrosis Score. The AUROCs for the various scores examined in this study, all performed similar to previous reports for the identification of advanced fibrosis[27,37–39]. In contrast, ADAPT was superior, as also in the multi-national validation cohort. Critically, ADAPT was robust at identifying patients with advanced fibrosis across different subpopulations (diabetics vs non-diabetics, NAFL vs NASH, various age ranges and BMI categories), some of which have been shown to confound non-invasive algorithms[40,41]. The AUROC of ADAPT was maintained at >0.80 for all subpopulations, while the PPV and NPV remained consistent. From a management perspective, after the application of a derived cut-off value, ADAPT correctly classified 74% of patients without advanced fibrosis and 92% with advanced fibrosis. Cut-off values for APRI, FIB-4 and NAFLD Fibrosis Score were applied to our patients; similar to previous reports, we found that a large proportion of patients fell within an indeterminate zone [37]. FIB-4 and APRI showed reasonable performance at identifying patients without advanced fibrosis, but performed poorly at identifying patients with advanced fibrosis. Furthermore, the superiority of ADAPT is exemplified by its robust performance across various sub-populations (supplementary table 1) and by the substantially higher NPV. In contrast, the performance of FIB-4 has been demonstrated to be variable and is affected by confounders such as age. Additionally, unlike FIB-4, ADAPT is unburdened by the presence of an intermediate zone, which hinders its accuracy[37,41–44]. An advantage of PRO-C3 used alone or in combination as in ADAPT, is that it may stratify cirrhosis since the score is on a spectrum. This contrasts with FIB-4 or the NAFLD fibrosis score which are based on a dichotomous threshold. Hence, PRO-C3 based scores may have potential in patient monitoring over time, though this needs validation.

In contrast to the other non-invasive scores, ADAPT is distinct in that it combines PRO-C3 with important clinical and metabolic parameters associated with disease severity. Both increased age and the presence of diabetes are well-established risk factors for progressive liver disease and are easily discerned[45]. Similarly, platelet count is routinely measured and is strongly correlated with liver fibrosis and has been incorporated into multiple other non-invasive scoring systems[10,12,14]. A study by Mofrad *et al* has shown that the full spectrum of liver fibrosis stages can be found in patients presenting with liver enzymes in the normal range[8]. In addition, liver enzymes are sensitive to age leading to false positive results. Thus, previous analysis has shown that FIB-4 (and likely also APRI and the NAFLD fibrosis score) cannot be universally applied without modification to all patient groups[41]. The lack of inclusion of liver enzymes in ADAPT is thus a conspicuous advantage.

Non-invasive tests have been proposed as screening tools for detecting advanced liver fibrosis in the general population, where the prevalence of this outcome is low[46]. Score systems such as ADAPT, that exhibit a high specificity and NPV could provide a useful tool for clinicians as they reduce any uncertainty surrounding the diagnosis and the number of follow-up assessments required[46]. We propose that the ADAPT score could be used as such a screening tool within the general population to identify patients at risk of or with advanced fibrosis, such that interventions could be applied and progression to cirrhosis perhaps mitigated. However, further validation in non-referral cohorts and demonstration of the cost-effectiveness of using PRO-C3 based score systems is first required.

Our study has some limitations that must be acknowledged. We included well-characterized biopsied patients from centers with an interest in studying NAFLD, therefore referral bias cannot be ruled out. Biopsies were read by an independent pathologist at each participating center using a well-defined and standardized score system. In our hands, the kappa value for assessing the severity of fibrosis has previously been shown to be good[47]. As previously described by Ratziu *et al*, liver biopsy as a diagnostic tool has several limitations including sampling bias[6]. However, all non-invasive diagnostic tools for fibrosis

assessment are benchmarked against the biopsy. Thus, the use of an imperfect reference standard may result in underperformance of the accuracy of non-invasive scores. Additionally, due to the nature of this cross-sectional study, we could not follow the clinical progress of patients; it would be of interest to investigate the relationship of score classification with patient outcome.

In conclusion, a biomarker score based on PRO-C3 and clinical variables (ADAPT) accurately predicts the presence or absence of advanced fibrosis in a NAFLD population. Thus, ADAPT could be useful for risk stratification and management. Further independent studies will be required to determine whether patients stratification using ADAPT followed by measurement of liver stiffness can replace the need for liver biopsy as a diagnostic standard in NAFLD.

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