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

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

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34 **List of Abbreviations**

35 ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs

36 ALT: Alanine Aminotransferase

37 APRI: AST to Platelet Ratio Index

38 AST: Aspartate Aminotransferase

39 AUROC: Area under receiver operating curve

40 BMI: Body mass index

41 ECM: Extracellular matrix

42 ELISA: Enzyme-linked immunosorbent assay

43 GGT: Gamma-Glutamyltransferase

44 HDL: High-density lipoprotein

45 LDL: Low-density lipoprotein

46 LHR+: Positive likelihood ratio

47 LHR-: Negative likelihood ratio

48 MS: Metabolic syndrome

49 NAFL: Non-alcoholic fatty liver

50 NAFLD: Non-alcoholic fatty liver disease

51 NASH: Non-alcoholic steatohepatitis

52 NFS: NAFLD Fibrosis Score

53 NPV: Negative predictive value

54 PPV: Positive predictive value

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58 Research Foundation supporting this work.

59 Abstract

60 Background and Aim: Given the high global prevalence of non-alcoholic fatty liver disease (NAFLD), the
61 need for relevant non-invasive biomarkers and algorithms to accurately stage disease severity is a critical
62 unmet medical need. Identifying those with advanced fibrosis ($\geq F3$) is the most crucial, as these individuals
63 have the greatest risk of adverse, long-term, liver-related outcomes. We aimed to investigate the role of
64 PRO-C3 (a marker of type III collagen formation) as a biomarker for advanced fibrosis in NAFLD. Methods:
65 We measured PRO-C3 by enzyme-linked immunosorbent assay (ELISA) in two large independent cohorts
66 with extensive clinical phenotyping and liver biopsy; 150 in the derivation and 281 in the validation cohort.
67 A PRO-C3 based fibrosis algorithm that included Age, presence of DiAbetes, PRO-C3 (a marker of type III
68 collagen formation), and plaTelet count ("ADAPT") was developed. Results: PRO-C3 increased with fibrosis
69 stage (ρ 0.50 $p < 0.0001$) and was independently associated with advanced fibrosis (OR=1.05, 95% CI 1.02-
70 1.08, $p = 0.003$). ADAPT showed areas under the receiver operating characteristics curve (AUROC) of 0.86
71 (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
72 fibrosis. This was superior to the existing fibrosis scores, aspartate aminotransferase (AST) to platelet ratio
73 index (APRI), FIB-4 and NAFLD fibrosis score (NFS) in most comparisons. Conclusion: PRO-C3 is an
74 independent predictor of fibrosis stage in NAFLD. A PRO-C3 based score (ADAPT) accurately identifies
75 patients with NAFLD and advanced fibrosis and is superior to APRI, FIB-4 and NFS.

76
77 Keywords

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

79 Introduction

80 The increase in global prevalence of metabolic syndrome (MS) has been accompanied by a rise in organ
81 damage including end stage disease related to non-alcoholic fatty liver disease (NAFLD). Estimates place the
82 worldwide prevalence of NAFLD at 25%¹. A subset of these patients develop non-alcoholic steatohepatitis
83 (NASH) that can progress to cirrhosis and are at a high risk of adverse liver-related outcomes¹. From a
84 management and therapeutic perspective, an unmet clinical need is the requirement to distinguish those
85 with early disease from those at highest risk of clinical complications. While metabolic hepatic
86 inflammation is the milieu that drives disease progression, various studies (including meta analyses) that
87 have examined for prognostic histological features suggest that fibrosis stage is the parameter that best
88 associates with overall- and liver-related mortality, as well as liver transplantation and liver related events²⁻
89 ⁵.

90 The gold standard for the evaluation of liver fibrosis stage is percutaneous needle biopsy, which is
91 compromised by inherent sampling and inter-observer biases and peri-procedural risk^{6,7}. The invasiveness
92 and costs of performing biopsies also makes it unsuitable for mass screening, for staging and risk
93 stratification. The latter is important as the majority of patients with advanced fibrosis and even cirrhosis,
94 are asymptomatic and often indistinguishable from those at earlier disease stages^{8,9}. In this context, there
95 is a need for surrogate markers of disease stage that can identify and risk stratify patients with NAFLD. This
96 area of research can broadly be divided into liquid (typically blood based) or physical approaches
97 (measurement of liver stiffness). Physical approaches while promising are less useful for population level
98 screening and are limited by cost and other technique-specific considerations. Several serum based
99 biomarker tests have previously been developed and applied to NAFLD patients¹⁰⁻¹⁴. These scores typically
100 combine clinical features and routine laboratory tests and are used primarily to rule out advanced fibrosis¹⁵,
101 however they lack sufficient diagnostic accuracy and sensitivity.

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6 102 Liver fibrosis is characterized by the accumulation of excess extracellular matrix (ECM) and hence
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8 103 biomarkers reflecting structural changes occurring in the hepatic ECM during chronic injury may be of value
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10 104 in the assessment of fibrosis progression or regression. We recently demonstrated that PRO-C3, an
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12 105 ADAMTS generated neo-epitope marker of type III collagen formation, is a marker of fibrosis in patients
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14 106 with chronic hepatitis C. The role of PRO-C3 in patients with NAFLD however, is largely unknown. Since the
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16 107 performance of biomarkers and non-invasive liver fibrosis scores varies widely according to disease etiology,
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18 108 whether PRO-C3 has a role as a biomarker in NAFLD is unclear. In this study, we sought to a) explore the
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20 109 association of PRO-C3 with liver fibrosis in two large independent biopsy-proven cohorts with NAFLD and b)
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22 110 determine if PRO-C3 can be combined with simple and routinely available clinical variables in to a novel
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24 111 score for the prediction of advanced fibrosis in patients with NAFLD. We compared the performance of our
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26 112 derived model with other known biomarker algorithms.

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5 115 **Materials and Methods**
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8 116 *Study population*
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11 117 A total of 431 well phenotyped patients with biopsy confirmed NAFLD comprised the study cohort. The
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13 118 derivation cohort included 150 patients from the Storr Liver Centre, Sydney, Australia; the validation cohort
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15 119 comprised 281 patients recruited from four international sites, Nottingham University Hospitals NHS Trust,
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17 120 United Kingdom (n=42); Kurume University School of Medicine, Kurume, Japan (n=48); University of
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19 121 Western Australia, Nedlands, Australia (n=144) and 47 additional patients from the Storr Liver Centre.
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22 122 All patients were referred for the investigation of abnormal liver tests or steatosis detected by ultrasound.
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24 123 The diagnosis of NAFLD was established by liver biopsy in all cases. Patients with disease of other etiologies
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26 124 including viral hepatitis and auto-immune liver disease were excluded by standard clinical, laboratory and
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28 125 histopathological assessments. Patients with evidence of hepatic decompensation, secondary causes of
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30 126 steatosis, including excess alcohol (men, >30 g/day; women, >20 g/day), total parenteral nutrition or the
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32 127 use of drugs known to precipitate steatosis were excluded.
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35 128 Demographic and clinical data were obtained, including age, gender, ethnicity, height, weight, and waist
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37 129 circumference at the time of biopsy. Body mass index (BMI) was calculated as $BMI = kg/m^2$. Arterial
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39 130 hypertension was defined as blood pressure $\geq 130/\geq 85$ mmHg or treatment with antihypertensive drugs.
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41 131 Diabetes was defined as a fasting blood glucose ≥ 7.0 mmol/L, previous diagnosis of diabetes or use of anti-
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43 132 diabetic drugs. Hyperlipidemia was defined as fasting total cholesterol >5.5 mmol/L, triglycerides >1.7
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45 133 mmol/L or treatment with lipid-lowering drugs. Ethical approval and written informed consent from
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47 134 patients was obtained from all participating centers.
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51 135 *Histology*
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5 136 All biopsies were routinely stained with hematoxylin & eosin and Masson's Trichrome. The stained sections
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7 137 were read and scored by an expert liver pathologist at each participating center using the scoring system
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9 138 proposed by Kleiner *et al.*, 2005¹⁶. The stage of liver fibrosis was defined as: stage 0, absence of fibrosis;
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11 139 stage 1, perisinusoidal or portal fibrosis; stage 2 perisinusoidal and portal/periportal fibrosis; stage 3 septal
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13 140 or bridging fibrosis; and stage 4 as cirrhosis. A diagnosis of NASH was according to the EASL-EASD-EASO
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15 141 guidelines.¹⁷ Thirty-one biopsies were scored independently by pathologists from the various centers, and
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17 142 inter observer agreement was calculated using the κ statistic and was =0.55 for fibrosis, comparable to
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19 143 previously published results¹⁸⁻²¹.

20 21 22 23 144 *Biomarker quantification*

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25 145 At the time of biopsy, a fasting blood sample was obtained and routine biochemical tests were performed
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27 146 using standard methods and assays. Biochemical tests included albumin, alanine aminotransferase (ALT),
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29 147 aspartate aminotransferase (AST), total cholesterol, gamma-glutamyltransferase (GGT), insulin, high-
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31 148 density lipoprotein (HDL), low-density lipoprotein (LDL), platelets, and triglycerides. Additional blood
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33 149 samples were drawn and frozen at -80°C for future research. Type III collagen formation was assessed in
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35 150 serum using the PRO-C3 competitive ELISA assay from Nordic Bioscience, Herlev, Denmark, as previously
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37 151 described²².

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41 152 The APRI, FIB-4 and NAFLD Fibrosis Scores were calculated using clinical and routine laboratory variables
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43 153 and previously defined algorithms and cut-off values for NAFLD/NASH patients^{10,12,14,23}.

44 45 46 154 *Statistical analysis*

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48 155 The main aim of this study was the development of an algorithm comprised of clinical and laboratory
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50 156 variables that could accurately distinguish patients with advanced fibrosis ($F \geq 3$) from those without. To this
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52 157 end, patients in the derivation cohort were stratified into those with advanced fibrosis ($F \geq 3$) and those
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54 158 without ($F 0-2$). Stage 2 and 3 for lobular and portal inflammation was pooled as only 1 patient in both
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5 159 groups was graded stage 3. Continuous variables in the two groups were compared using the t test and
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7 160 categorical variables were compared using Fisher's exact test. Comparisons between mean marker levels
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9 161 were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Variables that
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11 162 were significantly different between patients with advanced fibrosis and those without advanced fibrosis
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13 163 were identified as potential algorithm components.

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16 164 For the formulation of predictive models, variables showing a $p < 0.05$ at univariate analysis (Student t test
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18 165 for parametric variables, and χ^2 or Fisher exact test for frequencies) were included. The interaction
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20 166 between these variables was first tested. Variables explaining a statistically significant proportion of the
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22 167 variance ($p < 0.05$) were maintained in the model using the likelihood ratio (LR) test. The model variables
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24 168 were selected using the leave-one-out method to facilitate the calculation of over-fit bias reduced
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26 169 estimates²⁴. To avoid over-fitting, 10-fold cross validations were used in the tree building process.

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30 170 The model was as following:

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

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36 171 The discriminative ability of the model for the identification of severe fibrosis ($F \geq 3$) was assessed by means
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38 172 of receiver operating characteristic curve analysis and expressed as area under the receiver operating
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40 173 characteristic curve (AUROC). A cut-off value to distinguish patients with advanced fibrosis from those
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42 174 without was determined using the bootstrap Youden Index. The diagnostic accuracy of the algorithm and
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44 175 the derived cut-off was determined by calculating sensitivity, specificity, positive predictive value (PPV) and
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46 176 negative predictive value (NPV). To overcome both spectrum effect and ordinal scale issues, we undertook
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48 177 two approaches. Firstly, we used the Obuchowski measure, as proposed by Lambert *et al*^{25,26}, which is a
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50 178 measure of the probability that two randomly chosen patients from different fibrosis stages are correctly
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52 179 classified according to the weighted scheme, with a penalty for incorrect classification. In the second
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5 180 method, we standardized the AUROC for the distribution of fibrosis stages as proposed by Poynard *et al*²⁷,
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7 181 as recently described²⁸.
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10 182 ROC curves were also calculated for the established diagnostic scores, APRI, FIB-4 and NAFLD Fibrosis Score.
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12 183 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative
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14 184 likelihood ratio (LHR^+ , LHR^-) and 95% CIs were calculated. Estimates of AUROCs and comparisons between
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16 185 AUROCs were performed using the method suggested by Hanley and McNeil²⁹. Validation was subsequently
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18 186 performed on the validation cohort as well as for the combined overall cohort.
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21 187 All data are shown as medians and variation expressed via Tukey plots. P-values <5% were considered
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23 188 significant. Model building and statistical analysis was performed using MedCalc version 16.8.4 (MedCalc
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25 189 Software, Ostend, Belgium) and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Graphs were designed
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27 190 using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA).
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5 191 **Results**

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8 192 *Patient Characteristics*

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11 193 The characteristics of the 150 NAFLD patients used to develop the model (derivation cohort) and the 281
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13 194 used to test the model (validation cohort) are shown in Table 1. Serum levels of albumin, AST, cholesterol
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15 195 and HDL were all significantly lower in the validation cohort when compared to the derivation cohort. In
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17 196 addition, both BMI and insulin level were found to be significantly elevated. No other significant differences
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19 197 were observed between the two cohorts. The prevalence of severe fibrosis was not significantly different
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21 198 between the cohorts.

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24 199 *PRO-C3 is highly associated with severity of fibrosis and histological parameters*

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27 200 Within the derivation cohort, a neo-epitope marker of type III collagen formation, PRO-C3, was found to be
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29 201 significantly elevated in patients with advanced fibrosis (F \geq 3) compared to the mild/moderate group
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31 202 (p<0.0001). PRO-C3 was highly associated with disease severity (Figure 1) and moderately correlated to the
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33 203 severity of fibrosis (rho = 0.501, p<0.0001). PRO-C3 was able to discriminate between the following stages
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35 204 of fibrosis (Figure 1): F0 versus F2 (27% increase, p<0.0332), F0 versus F3 (54% increase, p<0.0001), F1
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37 205 versus F3 (36% increase, p<0.0002) and F0 versus F4 (57% increase, p<0.0021). In addition, PRO-C3
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39 206 discriminated between the various stages of hepatocyte ballooning (stage 0 versus stage 1 p=0.001, stage 0
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41 207 versus 2 p=0.0003), lobular inflammation (stage 0 versus stage 1 p=0.0004, stage 0 versus stage 2 and 3
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43 208 p=0.0008) and steatosis (stage 1 versus stage 3 p=0.003).

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46 209 We undertook logistic regression to discern the effect of various clinical variables on the association of
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48 210 PRO-C3 with the presence of advanced fibrosis (F \geq 3) within the derivation cohort. In this analysis, PRO-C3,
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50 211 when adjusted for age, ALT, AST, BMI, ballooning, lobular inflammation, presence of diabetes, GGT and
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52 212 platelet count, was independently associated with advanced fibrosis (OR=1.054, 95% CI 1.01-1.07) (Table 2).

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5 213 The area under the receiver-operating curve (AUROC) for the identification of patients with advanced
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7 214 fibrosis (F \geq 3) of PRO-C3 alone was 0.81 (95% CI 0.74-0.87) (Data not shown).
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10 215 *Clinical parameters associated with the level of PRO-C3*
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13 216 Given that univariate and multivariate analyses revealed that the level of PRO-C3 was a strong predictor of
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15 217 advanced fibrosis, we examined for clinical parameters associated with the level of PRO-C3. It was
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17 218 subsequently found that ALT (rho 0.29, p=0.0004), AST (rho 0.42, p<0.0001), fasting blood glucose (rho 0.23,
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19 219 p=0.007), insulin level (rho 0.43, p<0.0001), platelet count (rho -0.24, p=0.004) and presence of diabetes
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21 220 (rho 0.16, p=0.05) all correlated with the level of PRO-C3 to varying degrees.
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27 222 *Development of a PRO-C3 based predictive fibrosis score (ADAPT)*
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30 223 Based on the finding that PRO-C3 is strongly associated with fibrosis, we sought to build a model for the
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32 224 prediction of significant fibrosis based on PRO-C3 and routinely assessed clinical and laboratory variables.
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34 225 Patients within the derivation cohort were divided into two groups according to NASH CRN fibrosis stage,
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36 226 F0-2 (no fibrosis to moderate fibrosis) and F3-4 (advanced fibrosis) (Table 3). PRO-C3 was elevated in
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38 227 patients with advanced fibrosis compared to the mild to moderate group (p<0.0001). Furthermore, those
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40 228 with advanced fibrosis had significantly increased levels of AST, GGT and a higher AST/ALT ratio (Table 3).
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42 229 As would be expected, patients with advanced fibrosis had a worse metabolic profile with lower LDL, higher
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44 230 circulating insulin levels and a higher waist-to-hip ratio (Table 3). The presence of diabetes was more likely
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46 231 in patients with advanced fibrosis; 67% of patients with F3-4 had diabetes compared to just 29% of the F0-2
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48 232 group (p=0.002) (Table 3). In addition, patients with advanced fibrosis were found to be older (p=0.02) and
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50 233 with a lower platelet count compared to those without (p=0.002) (Table 3).
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5 234 Variables that were significantly different between the two groups ($p < 0.05$) were considered eligible for the
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7 235 model building process. Those that described a statistically significant proportion of the variance were
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9 236 included in the model using the likelihood ratio (LR) test. Ultimately, the variables that were included within
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11 237 the model, named "ADAPT", were age, presence of diabetes, platelet count and PRO-C3.

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14 238 The diagnostic capability of the ADAPT score was assessed via AUROC and was higher than that of PRO-C3
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16 239 alone, yielding an AUROC of 0.86 (95% CI 0.79-0.91) (Figure 2).

17 18 19 240 *Validation of the diagnostic capabilities of the ADAPT score*

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21 241 To ascertain the validity of our model, the ability of ADAPT to identify patients with advanced fibrosis was
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23 242 corroborated in a separate cohort comprised of patients from four centers across Asia-Pacific and Europe
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25 243 ($n=281$). Several significant differences were identified between the derivation and the validation cohort;
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27 244 these differences reflect the heterogeneity of NAFLD patients with advanced fibrosis. Despite cohort
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29 245 differences, the diagnostic accuracy of ADAPT was maintained with an AUROC in the validation cohort of
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31 246 0.87 (95% CI 0.83-0.91) (Figure 3).

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34 247 The diagnostic performance of a score, when assessed by AUROC, may vary according to disease
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36 248 prevalence, known as spectrum bias³⁰. The Obuchowski measure accounts for the spectrum bias and
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38 249 provides a means by which the diagnostic accuracy of a score can be assessed. The Obuchowski measure of
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40 250 ADAPT within the derivation cohort was calculated to be 0.86 and within the validation cohort it was 0.89.
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42 251 Additionally, we standardized the AUROC for the distribution of fibrosis stages according to Poynard *et al*²⁷.
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44 252 The standardized AUROC of ADAPT was found to be 0.89 and 0.89 within the derivation and validation
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46 253 cohorts, respectively (Table 4). For further confirmation of the generalizability of the model, the validation
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48 254 cohort was stratified into various groups according to age, (<50, 50-60 and >60), BMI, Sex, NASH vs NAFL
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50 255 and center. In this analysis, ADAPT remained a robust algorithm in that the AUROC was maintained across
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52 256 all sub-populations, with NPV consistently exceeding 90% (Supplementary Figure 1).

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5 257 *Performance of ADAPT against standard algorithms*
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8 258 Within the derivation cohort the AUROC of “ADAPT” (AUROC=0.855) was superior to clinically available
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10 259 serum based non-invasive scores: APRI (AUROC=0.73, p=0.02), FIB-4 (AUROC=0.78, p=0.06) and NAFLD
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12 260 Fibrosis Score (AUROC=0.78, p=0.06) (Table 4). Likewise, in the validation cohort, the AUROC of “ADAPT”
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14 261 (AUROC=0.87) was greater than APRI (AUROC=0.78, p=0.0005), FIB-4 (AUROC=0.85, p=0.32) and NAFLD
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16 262 Fibrosis Score (AUROC= 0.79, p=0.02) (Table 4). Adjusting the AUROC according to Poynard *et al*²⁷ caused
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18 263 minor increases in the AUROC in all scores (Table 4). Further investigation into the ability of ADAPT to
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20 264 identify patients with clinically significant fibrosis (F2-F4) highlighted the superiority of the ADAPT score
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22 265 when compared to other clinically available serum based non-invasive scores (supplementary table 3).
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26 266 *Derivation of cut-off values*
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28 267 The derivation cohort was subjected to ROC curve analysis to derive a cut-off value for the rule-in and rule-
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30 268 out of advanced fibrosis. A value of >6.3287 for the rule in/out of advanced fibrosis was identified by the
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32 269 Youden Index, PPV 48.4%, NPV 96.6%, (Supplementary table 2). By applying this cut-off, 73% (n=158) F0-2
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34 270 patients were correctly classified and 27% (n=58) incorrectly classified. Among F3-4 patients, 92% (n=60)
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36 271 were correctly classified while 8% (n=5) were incorrectly classified (Table 5). We applied previously derived
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38 272 cut-off values for APRI (rule in advanced fibrosis >1.5, rule out advanced fibrosis <0.5), FIB-4 (rule in
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40 273 advanced fibrosis >2.67, rule out advanced fibrosis <1.3) and NAFLD Fibrosis Score (rule in advanced
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42 274 fibrosis >0.676, rule out advanced fibrosis <-1.455)^{10,14,23}. A large proportion of patients fell within an
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44 275 indeterminate zone, table 5. FIB-4 and APRI showed reasonable performance at identifying patients
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46 276 without advanced liver fibrosis, 68% (n=147) and 67% (n=145) were correctly classified, respectively.
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48 277 However, these scores performed poorly at identifying patients with advanced liver fibrosis, NAFLD Fibrosis
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50 278 Score and FIB-4 correctly identified 51% (n=33) and 46% (n=30) patients, respectively.
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279 Discussion

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

287 Non-invasive biomarkers that reflect the process of hepatic fibrosis are urgently needed; collagen
288 formation biomarkers are thus attractive targets. Here we demonstrate that PRO-C3, which measures type
289 III collagen synthesis is a novel and precise marker for advanced liver fibrosis in concordance with what we
290 have recently shown in chronic hepatitis C^{22,31,32}. Notably, a recent small non-biopsy study (n=297) from a
291 phase III study of balaglitazone in patients with late-stage Type 2 diabetes (BALLEET study) suggested that
292 PRO-C3 could have utility as a determinant of treatment response to a potential anti-fibrotic therapy³³.
293 Karsdal et al (2016) subsequently confirmed this within a study investigating the anti-fibrotic efficacy of
294 farglitazar³³; Harrison et al (2018) further explored PRO-C3 as a determinant of treatment response within a
295 phase IIb study³⁴. Though that finding needs to be validated in biopsy proven cohorts, their data in
296 combination with our findings suggest that PRO-C3 could serve as a biomarker not only for prediction of
297 fibrosis progression, but also for treatment response. Interestingly, the optimal cut off value in our study
298 was 15.6 ng/ml for advanced fibrosis, which is significantly different from that in patients with hepatitis C
299 (20 ng/ml)³⁵. Consistently, the cut off level for PRO-C3 was also lower in the BALLEET report (13.1 ng/ml)³³.
300 Further studies will be required to confirm the optimal cut off in NAFLD. It is noteworthy that the levels of
301 PRO-C3 did not increase from F3 to F4. The explanation for this finding is not clear and further mechanistic
302 studies are required.

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5 303 Previous reports have suggested that the pro-peptide of type III collagen^{22,36} can be used as a biomarker for
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7 304 NASH. However, we have shown that PRO-C3 is distinct from PIIINP in that it is a true marker of type III
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9 305 collagen formation and by extension, fibrogenesis²². We subsequently developed a novel PRO-C3-based
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11 306 fibrosis score for NAFLD patients and compared it to various composite serum based score systems that
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13 307 have been proposed and tested in NAFLD patients, namely APRI, FIB-4 and the NAFLD Fibrosis Score. The
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15 308 AUROCs for the various scores examined in this study, all performed similar to previous reports for the
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17 309 identification of advanced fibrosis^{27,37-39}. In contrast, ADAPT was superior, as also in the multi-national
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19 310 validation cohort. Critically, ADAPT was robust at identifying patients with advanced fibrosis across
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21 311 different subpopulations (diabetics vs non-diabetics, NAFL vs NASH, various age ranges and BMI categories),
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23 312 some of which have been shown to confound non-invasive algorithms^{40,41}. The AUROC of ADAPT was
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25 313 maintained at >0.80 for all subpopulations, while the PPV and NPV remained consistent. From a
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27 314 management perspective, after the application of a derived cut-off value, ADAPT correctly classified 74% of
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29 315 patients without advanced fibrosis and 92% with advanced fibrosis. Cut-off values for APRI, FIB-4 and
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31 316 NAFLD Fibrosis Score were applied to our patients; similar to previous reports, we found that a large
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33 317 proportion of patients fell within an indeterminate zone³⁷. FIB-4 and APRI showed reasonable performance
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35 318 at identifying patients without advanced fibrosis, but performed poorly at identifying patients with
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37 319 advanced fibrosis. Furthermore, the superiority of ADAPT is exemplified by its robust performance across
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39 320 various sub-populations (supplementary table 1) and by the substantially higher NPV. In contrast, the
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41 321 performance of FIB-4 has been demonstrated to be variable and is affected by confounders such as age.
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43 322 Additionally, unlike FIB-4, ADAPT is unburdened by the presence of an intermediate zone, which hinders its
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45 323 accuracy^{37,41-44}. An advantage of PRO-C3 used alone or in combination as in ADAPT, is that it may stratify
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47 324 cirrhosis since the score is on a spectrum. This contrasts with FIB-4 or the NAFLD fibrosis score which are
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49 325 based on a dichotomous threshold. Hence, PRO-C3 based scores may have potential in patient monitoring
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51 326 over time, though this needs validation.
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5 327 In contrast to the other non-invasive scores, ADAPT is distinct in that it combines PRO-C3 with important
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7 328 clinical and metabolic parameters associated with disease severity. Both increased age and the presence of
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9 329 diabetes are well-established risk factors for progressive liver disease and are easily discerned⁴⁵. Similarly,
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11 330 platelet count is routinely measured and is strongly correlated with liver fibrosis and has been incorporated
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13 331 into multiple other non-invasive scoring systems^{10,12,14}. A study by Mofrad *et al* has shown that the full
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15 332 spectrum of liver fibrosis stages can be found in patients presenting with liver enzymes in the normal
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17 333 range⁸. In addition, liver enzymes are sensitive to age leading to false positive results. Thus, previous
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19 334 analysis has shown that FIB-4 (and likely also APRI and the NAFLD fibrosis score) cannot be universally
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21 335 applied without modification to all patient groups⁴¹. The lack of inclusion of liver enzymes in ADAPT is thus
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23 336 a conspicuous advantage.

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27 337 Non-invasive tests have been proposed as screening tools for detecting advanced liver fibrosis in the
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29 338 general population, where the prevalence of this outcome is low⁴⁶. Score systems such as ADAPT, that
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31 339 exhibit a high specificity and NPV could provide a useful tool for clinicians as they reduce any uncertainty
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33 340 surrounding the diagnosis and the number of follow-up assessments required⁴⁶. We propose that the
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35 341 ADAPT score could be used as such a screening tool within the general population to identify patients at
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37 342 risk of or with advanced fibrosis, such that interventions could be applied and progression to cirrhosis
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39 343 perhaps mitigated. However, further validation in non-referral cohorts and demonstration of the cost-
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41 344 effectiveness of using PRO-C3 based score systems is first required.

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44 345 Our study has some limitations that must be acknowledged. We included well-characterized biopsied
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46 346 patients from centers with an interest in studying NAFLD, therefore referral bias cannot be ruled out.
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48 347 Biopsies were read by an independent pathologist at each participating center using a well-defined and
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50 348 standardized score system. In our hands, the kappa value for assessing the severity of fibrosis has
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52 349 previously been shown to be good⁴⁷. As previously described by Ratziu *et al*, liver biopsy as a diagnostic tool
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54 350 has several limitations including sampling bias⁶. However, all non-invasive diagnostic tools for fibrosis
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5 351 assessment are benchmarked against the biopsy. Thus, the use of an imperfect reference standard may
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7 352 result in underperformance of the accuracy of non-invasive scores. Additionally, due to the nature of this
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9 353 cross-sectional study, we could not follow the clinical progress of patients; it would be of interest to
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11 354 investigate the relationship of score classification with patient outcome.
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14 355 In conclusion, a biomarker score based on PRO-C3 and clinical variables (ADAPT) accurately predicts the
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16 356 presence or absence of advanced fibrosis in a NAFLD population. Thus, ADAPT could be useful for risk
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18 357 stratification and management. Further independent studies will be required to determine whether
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20 358 patients stratification using ADAPT followed by measurement of liver stiffness can replace the need for liver
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22 359 biopsy as a diagnostic standard in NAFLD.
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465 Author names in bold designate shared co-first authorship

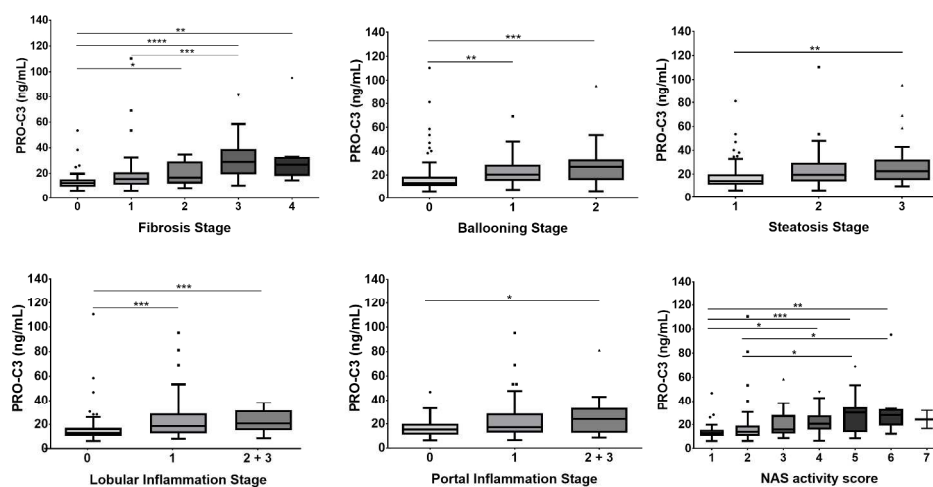


Figure 1: PRO-C3 is highly related to histological parameters of NAFLD!! † Histological staging according to Kleiner et al 16. Fibrosis stage ($\rho=0.50$, $p<0.0001$), Ballooning stage ($\rho=0.38$, $p<0.0001$), Steatosis stage ($\rho=0.29$, $p=0.0003$), Lobular inflammation ($\rho=0.36$, $p<0.0001$), 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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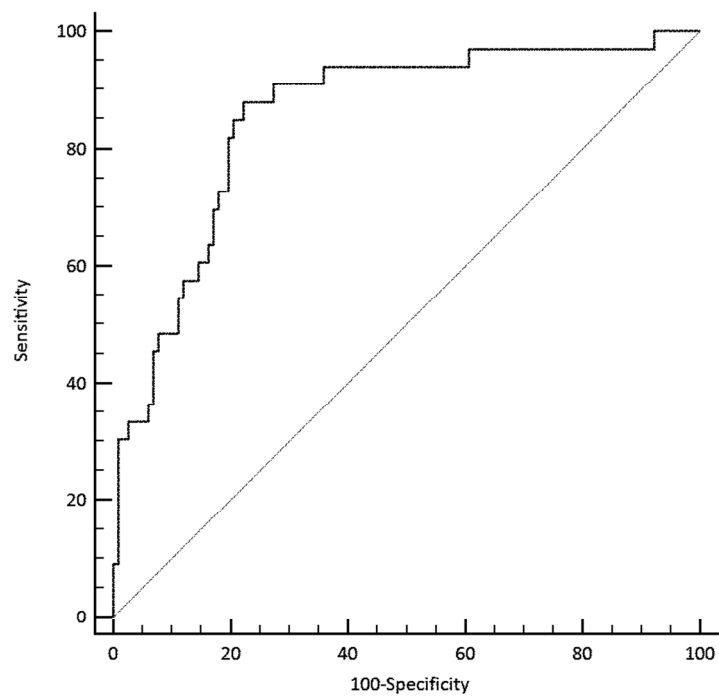


Figure 2: Receiver operating curve (ROC) for the identification of advanced fibrosis by ADAPT within the derivation cohort. AUROC = 0.86 (95% CI 0.79 to 0.91).

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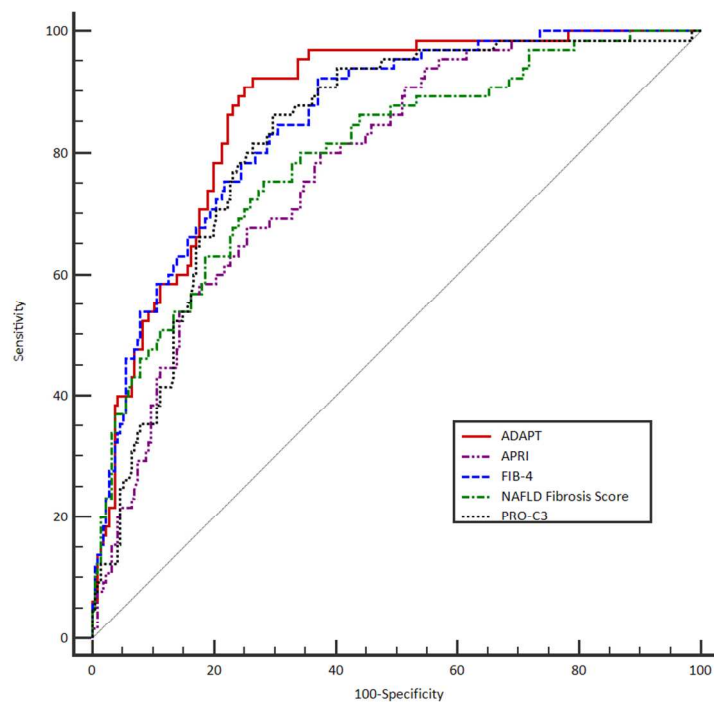


Figure 3: Comparison of various non-invasive serum based scores for the detection of advanced fibrosis (F≥3).

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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

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4	Platelets ($\times 10^9/L$)	148	244.4	73.53	270	229.7	79.49	ns
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6	PRO-C3 (ng/mL)	150	20.92	15.48	279	19.93	18.04	ns
7								
8								
9	Triglycerides (mmol/L)	149	2.03	1.60	263	1.97	1.40	ns
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11	Waist/Hip ratio	136	0.97	0.08	41	0.97	0.09	ns
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For Peer Review

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			p ^a
	n	Mean	SD	n	Mean	SD	
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

Non-invasive test	Derivation Cohort				Validation Cohort			
	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		Incorrectly	Correctly		Incorrectly
	Identified	Indeterminate	Identified	Identified	Indeterminate	Identified
Σ		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

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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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6 1 Manuscript number: HEP-18-0074

7 2 ADAPT: An algorithm incorporating PRO-C3 accurately identifies patients with
8 3 NAFLD and advanced fibrosis
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5 34 **List of Abbreviations**
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7 35 ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs
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9 36 ALT: Alanine Aminotransferase
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11 37 APRI: AST to Platelet Ratio Index
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13 38 AST: Aspartate Aminotransferase
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15 39 AUROC: Area under receiver operating curve
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17 40 BMI: Body mass index
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19 41 ECM: Extracellular matrix
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21 42 ELISA: Enzyme-linked immunosorbent assay
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23 43 GGT: Gamma-Glutamyltransferase
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25 44 HDL: High-density lipoprotein
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27 45 LDL: Low-density lipoprotein
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29 46 LHR+: Positive likelihood ratio
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31 47 LHR-: Negative likelihood ratio
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33 48 MS: Metabolic syndrome
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35 49 NAFL: Non-alcoholic fatty liver
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37 50 NAFLD: Non-alcoholic fatty liver disease
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39 51 NASH: Non-alcoholic steatohepatitis
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41 52 NFS: NAFLD Fibrosis Score
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43 53 NPV: Negative predictive value
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45 54 PPV: Positive predictive value
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51 58 Research Foundation supporting this work.
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59 Abstract

60 Background and Aim: Given the high global prevalence of non-alcoholic fatty liver disease (NAFLD), the
61 need for relevant non-invasive biomarkers and algorithms to accurately stage disease severity is a critical
62 unmet medical need. Identifying those with advanced fibrosis ($\geq F3$) is the most crucial, as these individuals
63 have the greatest risk of adverse, long-term, liver-related outcomes. We aimed to investigate the role of
64 PRO-C3 (a marker of type III collagen formation) as a biomarker for advanced fibrosis in NAFLD. Methods:
65 We measured PRO-C3 by enzyme-linked immunosorbent assay (ELISA) in two large independent cohorts
66 with extensive clinical phenotyping and liver biopsy; 150 in the derivation and 281 in the validation cohort.
67 A PRO-C3 based fibrosis algorithm that included Age, presence of DiAbetes, PRO-C3 (a marker of type III
68 collagen formation), and plaTelet count ("ADAPT") was developed. Results: PRO-C3 increased with fibrosis
69 stage (ρ 0.50 $p < 0.0001$) and was independently associated with advanced fibrosis (OR=1.05, 95% CI 1.02-
70 1.08, $p = 0.003$). ADAPT showed areas under the receiver operating characteristics curve (AUROC) of 0.86
71 (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
72 fibrosis. This was superior to the existing fibrosis scores, aspartate aminotransferase (AST) to platelet ratio
73 index (APRI), FIB-4 and NAFLD fibrosis score (NFS) in most comparisons. Conclusion: PRO-C3 is an
74 independent predictor of fibrosis stage in NAFLD. A PRO-C3 based score (ADAPT) accurately identifies
75 patients with NAFLD and advanced fibrosis and is superior to APRI, FIB-4 and NFS.

76
77 Keywords

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

79 Introduction

80 The increase in global prevalence of metabolic syndrome (MS) has been accompanied by a rise in organ
81 damage including end stage disease related to non-alcoholic fatty liver disease (NAFLD). Estimates place the
82 worldwide prevalence of NAFLD at 25%¹. A subset of these patients develop non-alcoholic steatohepatitis
83 (NASH) that can progress to cirrhosis and are at a high risk of adverse liver-related outcomes¹. From a
84 management and therapeutic perspective, an unmet clinical need is the requirement to distinguish those
85 with early disease from those at highest risk of clinical complications. While metabolic hepatic
86 inflammation is the milieu that drives disease progression, various studies (including meta analyses) that
87 have examined for prognostic histological features suggest that fibrosis stage is the parameter that best
88 associates with overall- and liver-related mortality, as well as liver transplantation and liver related events²⁻
89 ⁵.

90 The gold standard for the evaluation of liver fibrosis stage is percutaneous needle biopsy, which is
91 compromised by inherent sampling and inter-observer biases and peri-procedural risk^{6,7}. The invasiveness
92 and costs of performing biopsies also makes it unsuitable for mass screening, for staging and risk
93 stratification. The latter is important as the majority of patients with advanced fibrosis and even cirrhosis,
94 are asymptomatic and often indistinguishable from those at earlier disease stages^{8,9}. In this context, there
95 is a need for surrogate markers of disease stage that can identify and risk stratify patients with NAFLD. This
96 area of research can broadly be divided into liquid (typically blood based) or physical approaches
97 (measurement of liver stiffness). Physical approaches while promising are less useful for population level
98 screening and are limited by cost and other technique-specific considerations. Several serum based
99 biomarker tests have previously been developed and applied to NAFLD patients¹⁰⁻¹⁴. These scores typically
100 combine clinical features and routine laboratory tests and are used primarily to rule out advanced fibrosis¹⁵,
101 however they lack sufficient diagnostic accuracy and sensitivity.

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6 102 Liver fibrosis is characterized by the accumulation of excess extracellular matrix (ECM) and hence
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8 103 biomarkers reflecting structural changes occurring in the hepatic ECM during chronic injury may be of value
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10 104 in the assessment of fibrosis progression or regression. We recently demonstrated that PRO-C3, an
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12 105 ADAMTS generated neo-epitope marker of type III collagen formation, is a marker of fibrosis in patients
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14 106 with chronic hepatitis C. The role of PRO-C3 in patients with NAFLD however, is largely unknown. Since the
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16 107 performance of biomarkers and non-invasive liver fibrosis scores varies widely according to disease etiology,
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18 108 whether PRO-C3 has a role as a biomarker in NAFLD is unclear. In this study, we sought to a) explore the
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20 109 association of PRO-C3 with liver fibrosis in two large independent biopsy-proven cohorts with NAFLD and b)
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22 110 determine if PRO-C3 can be combined with simple and routinely available clinical variables in to a novel
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24 111 score for the prediction of advanced fibrosis in patients with NAFLD. We compared the performance of our
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26 112 derived model with other known biomarker algorithms.
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5 115 **Materials and Methods**
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8 116 *Study population*
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11 117 A total of 431 well phenotyped patients with biopsy confirmed NAFLD comprised the study cohort. The
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13 118 derivation cohort included 150 patients from the Storr Liver Centre, Sydney, Australia; the validation cohort
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15 119 comprised 281 patients recruited from four international sites, Nottingham University Hospitals NHS Trust,
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17 120 United Kingdom (n=42); Kurume University School of Medicine, Kurume, Japan (n=48); University of
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19 121 Western Australia, Nedlands, Australia (n=144) and 47 additional patients from the Storr Liver Centre.
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22 122 All patients were referred for the investigation of abnormal liver tests or steatosis detected by ultrasound.
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24 123 The diagnosis of NAFLD was established by liver biopsy in all cases. Patients with disease of other etiologies
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26 124 including viral hepatitis and auto-immune liver disease were excluded by standard clinical, laboratory and
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28 125 histopathological assessments. Patients with evidence of hepatic decompensation, secondary causes of
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30 126 steatosis, including excess alcohol (men, >30 g/day; women, >20 g/day), total parenteral nutrition or the
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32 127 use of drugs known to precipitate steatosis were excluded.
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35 128 Demographic and clinical data were obtained, including age, gender, ethnicity, height, weight, and waist
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37 129 circumference at the time of biopsy. Body mass index (BMI) was calculated as $BMI = kg/m^2$. Arterial
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39 130 hypertension was defined as blood pressure $\geq 130/\geq 85$ mmHg or treatment with antihypertensive drugs.
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41 131 Diabetes was defined as a fasting blood glucose ≥ 7.0 mmol/L, previous diagnosis of diabetes or use of anti-
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43 132 diabetic drugs. Hyperlipidemia was defined as fasting total cholesterol >5.5 mmol/L, triglycerides >1.7
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45 133 mmol/L or treatment with lipid-lowering drugs. Ethical approval and written informed consent from
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47 134 patients was obtained from all participating centers.
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51 135 *Histology*
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5 136 All biopsies were routinely stained with hematoxylin & eosin and Masson's Trichrome. The stained sections
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7 137 were read and scored by an expert liver pathologist at each participating center using the scoring system
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9 138 proposed by Kleiner *et al.*, 2005¹⁶. The stage of liver fibrosis was defined as: stage 0, absence of fibrosis;
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11 139 stage 1, perisinusoidal or portal fibrosis; stage 2 perisinusoidal and portal/periportal fibrosis; stage 3 septal
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13 140 or bridging fibrosis; and stage 4 as cirrhosis. A diagnosis of NASH was according to the EASL-EASD-EASO
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15 141 guidelines.¹⁷ Thirty-one biopsies were scored independently by pathologists from the various centers, and
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17 142 inter observer agreement was calculated using the κ statistic and was =0.55 for fibrosis, comparable to
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19 143 previously published results¹⁸⁻²¹.

20 21 22 23 144 *Biomarker quantification*

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25 145 At the time of biopsy, a fasting blood sample was obtained and routine biochemical tests were performed
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27 146 using standard methods and assays. Biochemical tests included albumin, alanine aminotransferase (ALT),
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29 147 aspartate aminotransferase (AST), total cholesterol, gamma-glutamyltransferase (GGT), insulin, high-
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31 148 density lipoprotein (HDL), low-density lipoprotein (LDL), platelets, and triglycerides. Additional blood
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33 149 samples were drawn and frozen at -80°C for future research. Type III collagen formation was assessed in
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35 150 serum using the PRO-C3 competitive ELISA assay from Nordic Bioscience, Herlev, Denmark, as previously
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37 151 described²².

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41 152 The APRI, FIB-4 and NAFLD Fibrosis Scores were calculated using clinical and routine laboratory variables
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43 153 and previously defined algorithms and cut-off values for NAFLD/NASH patients^{10,12,14,23}.

44 45 46 154 *Statistical analysis*

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48 155 The main aim of this study was the development of an algorithm comprised of clinical and laboratory
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50 156 variables that could accurately distinguish patients with advanced fibrosis ($F \geq 3$) from those without. To this
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52 157 end, patients in the derivation cohort were stratified into those with advanced fibrosis ($F \geq 3$) and those
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54 158 without ($F 0-2$). Stage 2 and 3 for lobular and portal inflammation was pooled as only 1 patient in both
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5 159 groups was graded stage 3. Continuous variables in the two groups were compared using the t test and
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7 160 categorical variables were compared using Fisher's exact test. Comparisons between mean marker levels
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9 161 were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Variables that
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11 162 were significantly different between patients with advanced fibrosis and those without advanced fibrosis
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13 163 were identified as potential algorithm components.

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16 164 For the formulation of predictive models, variables showing a $p < 0.05$ at univariate analysis (Student t test
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18 165 for parametric variables, and χ^2 or Fisher exact test for frequencies) were included. The interaction
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20 166 between these variables was first tested. Variables explaining a statistically significant proportion of the
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22 167 variance ($p < 0.05$) were maintained in the model using the likelihood ratio (LR) test. The model variables
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24 168 were selected using the leave-one-out method to facilitate the calculation of over-fit bias reduced
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26 169 estimates²⁴. To avoid over-fitting, 10-fold cross validations were used in the tree building process.

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30 170 The model was as following:

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

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36 171 The discriminative ability of the model for the identification of severe fibrosis ($F \geq 3$) was assessed by means
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38 172 of receiver operating characteristic curve analysis and expressed as area under the receiver operating
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40 173 characteristic curve (AUROC). A cut-off value to distinguish patients with advanced fibrosis from those
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42 174 without was determined using the bootstrap Youden Index. The diagnostic accuracy of the algorithm and
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44 175 the derived cut-off was determined by calculating sensitivity, specificity, positive predictive value (PPV) and
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46 176 negative predictive value (NPV). To overcome both spectrum effect and ordinal scale issues, we undertook
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48 177 two approaches. Firstly, we used the Obuchowski measure, as proposed by Lambert *et al*^{25,26}, which is a
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50 178 measure of the probability that two randomly chosen patients from different fibrosis stages are correctly
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52 179 classified according to the weighted scheme, with a penalty for incorrect classification. In the second
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5 180 method, we standardized the AUROC for the distribution of fibrosis stages as proposed by Poynard *et al*²⁷,
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7 181 as recently described²⁸.
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10 182 ROC curves were also calculated for the established diagnostic scores, APRI, FIB-4 and NAFLD Fibrosis Score.
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12 183 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative
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14 184 likelihood ratio (LHR^+ , LHR^-) and 95% CIs were calculated. Estimates of AUROCs and comparisons between
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16 185 AUROCs were performed using the method suggested by Hanley and McNeil²⁹. Validation was subsequently
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18 186 performed on the validation cohort as well as for the combined overall cohort.
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21 187 All data are shown as medians and variation expressed via Tukey plots. P-values <5% were considered
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23 188 significant. Model building and statistical analysis was performed using MedCalc version 16.8.4 (MedCalc
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25 189 Software, Ostend, Belgium) and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Graphs were designed
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27 190 using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA).
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5 191 **Results**

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8 192 *Patient Characteristics*

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11 193 The characteristics of the 150 NAFLD patients used to develop the model (derivation cohort) and the 281
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13 194 used to test the model (validation cohort) are shown in Table 1. Serum levels of albumin, AST, cholesterol
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15 195 and HDL were all significantly lower in the validation cohort when compared to the derivation cohort. In
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17 196 addition, both BMI and insulin level were found to be significantly elevated. No other significant differences
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19 197 were observed between the two cohorts. The prevalence of severe fibrosis was not significantly different
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21 198 between the cohorts.

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24 199 *PRO-C3 is highly associated with severity of fibrosis and histological parameters*

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27 200 Within the derivation cohort, a neo-epitope marker of type III collagen formation, PRO-C3, was found to be
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29 201 significantly elevated in patients with advanced fibrosis (F \geq 3) compared to the mild/moderate group
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31 202 (p<0.0001). PRO-C3 was highly associated with disease severity (Figure 1) and moderately correlated to the
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33 203 severity of fibrosis (rho = 0.501, p<0.0001). PRO-C3 was able to discriminate between the following stages
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35 204 of fibrosis (Figure 1): F0 versus F2 (27% increase, p<0.0332), F0 versus F3 (54% increase, p<0.0001), F1
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37 205 versus F3 (36% increase, p<0.0002) and F0 versus F4 (57% increase, p<0.0021). In addition, PRO-C3
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39 206 discriminated between the various stages of hepatocyte ballooning (stage 0 versus stage 1 p=0.001, stage 0
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41 207 versus 2 p=0.0003), lobular inflammation (stage 0 versus stage 1 p=0.0004, stage 0 versus stage 2 and 3
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43 208 p=0.0008) and steatosis (stage 1 versus stage 3 p=0.003).

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46 209 We undertook logistic regression to discern the effect of various clinical variables on the association of
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48 210 PRO-C3 with the presence of advanced fibrosis (F \geq 3) within the derivation cohort. In this analysis, PRO-C3,
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50 211 when adjusted for age, ALT, AST, BMI, ballooning, lobular inflammation, presence of diabetes, GGT and
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52 212 platelet count, was independently associated with advanced fibrosis (OR=1.054, 95% CI 1.01-1.07) (Table 2).

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5 213 The area under the receiver-operating curve (AUROC) for the identification of patients with advanced
6 214 fibrosis ($F \geq 3$) of PRO-C3 alone was 0.81 (95% CI 0.74-0.87) (Data not shown).

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10 215 *Clinical parameters associated with the level of PRO-C3*

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13 216 Given that univariate and multivariate analyses revealed that the level of PRO-C3 was a strong predictor of
14 217 advanced fibrosis, we examined for clinical parameters associated with the level of PRO-C3. It was
15 218 subsequently found that ALT (ρ 0.29, $p=0.0004$), AST (ρ 0.42, $p<0.0001$), fasting blood glucose (ρ 0.23,
16 219 $p=0.007$), insulin level (ρ 0.43, $p<0.0001$), platelet count (ρ -0.24, $p=0.004$) and presence of diabetes
20 220 (ρ 0.16, $p=0.05$) all correlated with the level of PRO-C3 to varying degrees.

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24 221 *Development of a PRO-C3 based predictive fibrosis score (ADAPT)*

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27 222 Based on the finding that PRO-C3 is strongly associated with fibrosis, we sought to build a model for the
28 223 prediction of significant fibrosis based on PRO-C3 and routinely assessed clinical and laboratory variables.
29 224 Patients within the derivation cohort were divided into two groups according to NASH CRN fibrosis stage,
30 225 F0-2 (no fibrosis to moderate fibrosis) and F3-4 (advanced fibrosis) (Table 3). PRO-C3 was elevated in
31 226 patients with advanced fibrosis compared to the mild to moderate group ($p<0.0001$). Furthermore, those
32 227 with advanced fibrosis had significantly increased levels of AST, GGT and a higher AST/ALT ratio (Table 3).
33 228 As would be expected, patients with advanced fibrosis had a worse metabolic profile with lower LDL, higher
34 229 circulating insulin levels and a higher waist-to-hip ratio (Table 3). The presence of diabetes was more likely
35 230 in patients with advanced fibrosis; 67% of patients with F3-4 had diabetes compared to just 29% of the F0-2
36 231 group ($p=0.002$) (Table 3). In addition, patients with advanced fibrosis were found to be older ($p=0.02$) and
37 232 with a lower platelet count compared to those without ($p=0.002$) (Table 3).

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42 233 Variables that were significantly different between the two groups ($p<0.05$) were considered eligible for the
43 234 model building process. Those that described a statistically significant proportion of the variance were

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6 235 included in the model using the likelihood ratio (LR) test. Ultimately, the variables that were included within
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8 236 the model, named “ADAPT”, were age, presence of diabetes, platelet count and PRO-C3.
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10 237 The diagnostic capability of the ADAPT score was assessed via AUROC and was higher than that of PRO-C3
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12 238 alone, yielding an AUROC of 0.86 (95% CI 0.79-0.91) (Figure 2).
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15 239 *Validation of the diagnostic capabilities of the ADAPT score*

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17 240 To ascertain the validity of our model, the ability of ADAPT to identify patients with advanced fibrosis was
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19 241 corroborated in a separate cohort comprised of patients from four centers across Asia-Pacific and Europe
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21 242 (n=281). Several significant differences were identified between the derivation and the validation cohort;
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23 243 these differences reflect the heterogeneity of NAFLD patients with advanced fibrosis. Despite cohort
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25 244 differences, the diagnostic accuracy of ADAPT was maintained with an AUROC in the validation cohort of
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27 245 0.87 (95% CI 0.83-0.91) (Figure 3).
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30 246 The diagnostic performance of a score, when assessed by AUROC, may vary according to disease
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32 247 prevalence, known as spectrum bias³⁰. The Obuchowski measure accounts for the spectrum bias and
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34 248 provides a means by which the diagnostic accuracy of a score can be assessed. The Obuchowski measure of
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36 249 ADAPT within the derivation cohort was calculated to be 0.86 and within the validation cohort it was 0.89.
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38 250 Additionally, we standardized the AUROC for the distribution of fibrosis stages according to Poynard *et al*²⁷.
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40 251 The standardized AUROC of ADAPT was found to be 0.89 and 0.89 within the derivation and validation
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42 252 cohorts, respectively (Table 4). For further confirmation of the generalizability of the model, the validation
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44 253 cohort was stratified into various groups according to age, (<50, 50-60 and >60), BMI, Sex, NASH vs NAFL
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46 254 and center. In this analysis, ADAPT remained a robust algorithm in that the AUROC was maintained across
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48 255 all sub-populations, with NPV consistently exceeding 90% (Supplementary Figure 1).
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51 256 *Performance of ADAPT against standard algorithms*

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5 257 Within the derivation cohort the AUROC of “ADAPT” (AUROC=0.855) was superior to clinically available
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7 258 serum based non-invasive scores: APRI (AUROC=0.73, p=0.02), FIB-4 (AUROC=0.78, p=0.06) and NAFLD
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9 259 Fibrosis Score (AUROC=0.78, p=0.06) (Table 4). Likewise, in the validation cohort, the AUROC of “ADAPT”
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11 260 (AUROC=0.87) was greater than APRI (AUROC=0.78, p=0.0005), FIB-4 (AUROC=0.85, p=0.32) and NAFLD
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13 261 Fibrosis Score (AUROC= 0.79, p=0.02) (Table 4). Adjusting the AUROC according to Poynard *et al*²⁷ caused
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15 262 minor increases in the AUROC in all scores (Table 4). Further investigation into the ability of ADAPT to
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17 263 identify patients with clinically significant fibrosis (F2-F4) highlighted the superiority of the ADAPT score
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19 264 when compared to other clinically available serum based non-invasive scores (supplementary table 3).

20 21 22 23 265 *Derivation of cut-off values*

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25 266 The derivation cohort was subjected to ROC curve analysis to derive a cut-off value for the rule-in and rule-
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27 267 out of advanced fibrosis. A value of >6.3287 for the rule in/out of advanced fibrosis was identified by the
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29 268 Youden Index, PPV 48.4%, NPV 96.6%, (Supplementary table 2). By applying this cut-off, 73% (n=158) F0-2
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31 269 patients were correctly classified and 27% (n=58) incorrectly classified. Among F3-4 patients, 92% (n=60)
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33 270 were correctly classified while 8% (n=5) were incorrectly classified (Table 5). We applied previously derived
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35 271 cut-off values for APRI (rule in advanced fibrosis >1.5, rule out advanced fibrosis <0.5), FIB-4 (rule in
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37 272 advanced fibrosis >2.67, rule out advanced fibrosis <1.3) and NAFLD Fibrosis Score (rule in advanced
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39 273 fibrosis >0.676, rule out advanced fibrosis <-1.455)^{10,14,23}. A large proportion of patients fell within an
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41 274 indeterminate zone, table 5. FIB-4 and APRI showed reasonable performance at identifying patients
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43 275 without advanced liver fibrosis, 68% (n=147) and 67% (n=145) were correctly classified, respectively.
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45 276 However, these scores performed poorly at identifying patients with advanced liver fibrosis, NAFLD Fibrosis
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47 277 Score and FIB-4 correctly identified 51% (n=33) and 46% (n=30) patients, respectively.
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278 Discussion

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

286 Non-invasive biomarkers that reflect the process of hepatic fibrosis are urgently needed; collagen
287 formation biomarkers are thus attractive targets. Here we demonstrate that PRO-C3, which measures type
288 III collagen synthesis is a novel and precise marker for advanced liver fibrosis in concordance with what we
289 have recently shown in chronic hepatitis C^{22,31,32}. Notably, a recent small non-biopsy study (n=297) from a
290 phase III study of balaglitazone in patients with late-stage Type 2 diabetes (BALLET study) suggested that
291 PRO-C3 could have utility as a determinant of treatment response to a potential anti-fibrotic therapy³³.
292 Karsdal et al (2016) subsequently confirmed this within a study investigating the anti-fibrotic efficacy of
293 farglitazar³³; Harrison et al (2018) further explored PRO-C3 as a determinant of treatment response within a
294 phase IIb study³⁴. Though that finding needs to be validated in biopsy proven cohorts, their data in
295 combination with our findings suggest that PRO-C3 could serve as a biomarker not only for prediction of
296 fibrosis progression, but also for treatment response. Interestingly, the optimal cut off value in our study
297 was 15.6 ng/ml for advanced fibrosis, which is significantly different from that in patients with hepatitis C
298 (20 ng/ml)³⁵. Consistently, the cut off level for PRO-C3 was also lower in the BALLET report (13.1 ng/ml)³³.
299 Further studies will be required to confirm the optimal cut off in NAFLD. It is noteworthy that the levels of
300 PRO-C3 did not increase from F3 to F4. The explanation for this finding is not clear and further mechanistic
301 studies are required.

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5 302 Previous reports have suggested that the pro-peptide of type III collagen^{22,36} can be used as a biomarker for
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7 303 NASH. However, we have shown that PRO-C3 is distinct from PIIINP in that it is a true marker of type III
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9 304 collagen formation and by extension, fibrogenesis²². We subsequently developed a novel PRO-C3-based
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11 305 fibrosis score for NAFLD patients and compared it to various composite serum based score systems that
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13 306 have been proposed and tested in NAFLD patients, namely APRI, FIB-4 and the NAFLD Fibrosis Score. The
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15 307 AUROCs for the various scores examined in this study, all performed similar to previous reports for the
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17 308 identification of advanced fibrosis^{27,37-39}. In contrast, ADAPT was superior, as also in the multi-national
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19 309 validation cohort. Critically, ADAPT was robust at identifying patients with advanced fibrosis across
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21 310 different subpopulations (diabetics vs non-diabetics, NAFL vs NASH, various age ranges and BMI categories),
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23 311 some of which have been shown to confound non-invasive algorithms^{40,41}. The AUROC of ADAPT was
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25 312 maintained at >0.80 for all subpopulations, while the PPV and NPV remained consistent. From a
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27 313 management perspective, after the application of a derived cut-off value, ADAPT correctly classified 74% of
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29 314 patients without advanced fibrosis and 92% with advanced fibrosis. Cut-off values for APRI, FIB-4 and
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31 315 NAFLD Fibrosis Score were applied to our patients; similar to previous reports, we found that a large
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33 316 proportion of patients fell within an indeterminate zone³⁷. FIB-4 and APRI showed reasonable performance
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35 317 at identifying patients without advanced fibrosis, but performed poorly at identifying patients with
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37 318 advanced fibrosis. Furthermore, the superiority of ADAPT is exemplified by its robust performance across
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39 319 various sub-populations (supplementary table 1) and by the substantially higher NPV. In contrast, the
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41 320 performance of FIB-4 has been demonstrated to be variable and is affected by confounders such as age.
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43 321 Additionally, unlike FIB-4, ADAPT is unburdened by the presence of an intermediate zone, which hinders its
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45 322 accuracy^{37,41-44}. An advantage of PRO-C3 used alone or in combination as in ADAPT, is that it may stratify
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47 323 cirrhosis since the score is on a spectrum. This contrasts with FIB-4 or the NAFLD fibrosis score which are
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49 324 based on a dichotomous threshold. Hence, PRO-C3 based scores may have potential in patient monitoring
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51 325 over time, though this needs validation.
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6 326 In contrast to the other non-invasive scores, ADAPT is distinct in that it combines PRO-C3 with important
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8 327 clinical and metabolic parameters associated with disease severity. Both increased age and the presence of
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10 328 diabetes are well-established risk factors for progressive liver disease and are easily discerned⁴⁵. Similarly,
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12 329 platelet count is routinely measured and is strongly correlated with liver fibrosis and has been incorporated
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14 330 into multiple other non-invasive scoring systems^{10,12,14}. A study by Mofrad *et al* has shown that the full
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16 331 spectrum of liver fibrosis stages can be found in patients presenting with liver enzymes in the normal
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18 332 range⁸. In addition, liver enzymes are sensitive to age leading to false positive results. Thus, previous
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20 333 analysis has shown that FIB-4 (and likely also APRI and the NAFLD fibrosis score) cannot be universally
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22 334 applied without modification to all patient groups⁴¹. The lack of inclusion of liver enzymes in ADAPT is thus
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24 335 a conspicuous advantage.

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27 336 Non-invasive tests have been proposed as screening tools for detecting advanced liver fibrosis in the
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29 337 general population, where the prevalence of this outcome is low⁴⁶. Score systems such as ADAPT, that
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31 338 exhibit a high specificity and NPV could provide a useful tool for clinicians as they reduce any uncertainty
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33 339 surrounding the diagnosis and the number of follow-up assessments required⁴⁶. We propose that the
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35 340 ADAPT score could be used as such a screening tool within the general population to identify patients at
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37 341 risk of or with advanced fibrosis, such that interventions could be applied and progression to cirrhosis
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39 342 perhaps mitigated. However, further validation in non-referral cohorts and demonstration of the cost-
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41 343 effectiveness of using PRO-C3 based score systems is first required.

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44 344 Our study has some limitations that must be acknowledged. We included well-characterized biopsied
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46 345 patients from centers with an interest in studying NAFLD, therefore referral bias cannot be ruled out.
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48 346 Biopsies were read by an independent pathologist at each participating center using a well-defined and
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50 347 standardized score system. In our hands, the kappa value for assessing the severity of fibrosis has
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52 348 previously been shown to be good⁴⁷. As previously described by Ratziu *et al*, liver biopsy as a diagnostic tool
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54 349 has several limitations including sampling bias⁶. However, all non-invasive diagnostic tools for fibrosis
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5 350 assessment are benchmarked against the biopsy. Thus, the use of an imperfect reference standard may
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7 351 result in underperformance of the accuracy of non-invasive scores. Additionally, due to the nature of this
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9 352 cross-sectional study, we could not follow the clinical progress of patients; it would be of interest to
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11 353 investigate the relationship of score classification with patient outcome.
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14 354 In conclusion, a biomarker score based on PRO-C3 and clinical variables (ADAPT) accurately predicts the
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16 355 presence or absence of advanced fibrosis in a NAFLD population. Thus, ADAPT could be useful for risk
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18 356 stratification and management. Further independent studies will be required to determine whether
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20 357 patients stratification using ADAPT followed by measurement of liver stiffness can replace the need for liver
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22 358 biopsy as a diagnostic standard in NAFLD.
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