Title

Among simple non-invasive scores, Pro-C3 and ADAPT best exclude advanced fibrosis in Asian patients with MAFLD

Short Title: Serum PRO-C3, ADAPT and fibrosis staging

Authors:

Liang-Jie Tang [1], Hong-Lei Ma [1], Mohammed Eslam [2], Grace Lai-Hung Wong [3, 4], Pei-Wu Zhu [5], Sui-Dan Chen [6], Diana Julie Leeming [7], Morten Karsdal [7], Gang Li [1], Ou-Yang Huang [1], Howard Ho-Wai Leung [8], Yu-Jie Zhou [9], Qian Feng [10], Pei Jiang [11], Li-Mei Gao [11], Christopher D. Byrne [12], Giovanni Targher [13], Jacob George [2], Vincent Wai-Sun Wong [3, 4, *], Ming-Hua Zheng [1, 14, 15, *]

Affiliations:

[1]MAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

[2]Storr Liver Centre, Westmead Institute for Medical Research, Westmead Hospital, Westmead, and University of Sydney, Sydney, Australia;

[3]Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China;

[4]State Key Laboratory of Digestive Disease, The Chinese University of Hong Kong, Hong Kong, China;

[5]Department of Laboratory Medicine, the First Affiliated Hospital of Wenzhou

Medical University, Wenzhou, China;

[6]Department of Pathology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

[7]Nordic Bioscience Biomarkers and Research A/S, Herlev, Denmark;

[8]Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong, China;

[9]Division of Gastroenterology and Hepatology, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Renji Hospital, School of Medicine, Shanghai Jiao Tong University; Shanghai Institute of Digestive Disease, Shanghai, China;

[10]Department of Gastroenterology, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China;

[11]Fosun Diagnostics (Shanghai) Co., Ltd, Shanghai, China;

[12]Southampton National Institute for Health Research Biomedical Research Centre, University Hospital Southampton, Southampton General Hospital, Southampton, UK;

[13]Section of Endocrinology, Diabetes and Metabolism, Department of Medicine, University and Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy;

[14]Institute of Hepatology, Wenzhou Medical University, Wenzhou, China;

[15]Key Laboratory of Diagnosis and Treatment for the Development of Chronic Liver Disease in Zhejiang Province, Wenzhou, China

***Corresponding author:**

Ming-Hua Zheng, MD, PhD

MAFLD Research Center, Department of Hepatology, the First Affiliated Hospital of Wenzhou Medical University; No. 2 Fuxue Lane, Wenzhou 325000, China.
E-mail: zhengmh@wmu.edu.cn; fax: (86) 577-55578522; tel: (86) 577-55579622.

Vincent Wai-Sun Wong, MD
Department of Medicine and Therapeutics, 9/F Prince of Wales Hospital, 30-32 Ngan Shing Street, Shatin, Hong Kong, China.
E-mail: wongv@cuhk.edu.hk; fax: (852) 2637 3852; tel: (852) 3505 1205.

Abbreviation list:

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AAR, aspartate aminotransferase to alanine aminotransferase ratio; APRI, aspartate aminotransferase-platelet ratio index; AUROC, area under the receiver operating characteristic curve; BMI, body mass index; CI, 95% confidence interval; DCA, decision curve analysis; FIB-4, fibrosis-4; MAFLD, metabolic dysfunction-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NFS, NAFLD fibrosis score; NPV, negative predictive value; OR, odds ratio; OR, odds ratio; PRO-C3, N-terminal propeptide of type 3 collagen; PPV, positive predictive value;

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CRediT authorship contribution statement

Liang-Jie Tang: Conceptualization, Investigation, Data curation, Writing - original draft. **Hong-Lei Ma**: Conceptualization, Writing - original draft. **Mohammed Eslam**: Investigation, Writing - review & editing. **Grace Lai-Hung Wong**: Investigation, Writing - review & editing. **Pei-Wu Zhu**: Data curation, Writing - review & editing. **Sui-Dan Chen**: Data curation, Writing - review & editing. **Diana Julie Leeming**: Investigation, Writing - review & editing. **Morten Karsdal**: Investigation, Writing - review & editing. **Gang Li**: Data curation, Writing - review & editing. **Ou-Yang**

Huang: Data curation, Writing - review & editing. **Howard Ho-Wai Leung**: Data curation, Writing - review & editing. **Yu-Jie Zhou**: Investigation, Writing - review & editing. **Qian Feng**: Data curation, Writing - review & editing. **Pei Jiang**: Investigation, Writing - review & editing. **Li-Mei Gao**: Investigation, Writing - review & editing. **Christopher D. Byrne**: Investigation, Writing - review & editing. **Giovanni Targher**: Investigation, Writing - review & editing. **Jacob George**: Conceptualization, Methodology, Writing - review & editing. **Vincent Wai-Sun Wong**: Conceptualization, Methodology, Investigation, Writing - review & editing, Project administration. **Ming-Hua Zheng**: Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing, Project administration. All authors contributed to the manuscript for important intellectual content and approved the final submission of the manuscript.

Potential competing interests:

Although the PRO-C3 ELISA was carried out at Nordic Biosciences under a research collaboration, we confirm that cohort generation, research conceptualization, analysis, and manuscript drafting were carried out independent of the Nordic Biosciences team. ADAPT is not developed as a proprietary test. The PRO-C3 ELISA is not currently commercially available, but can be obtained as a Nordic Bioscience research test for research use only. Diana Julie Leeming is employed by, and owns stock in Nordic Bioscience. Pei Jiang and Li-Mei Gao are employed by Fosun Diagnostics (Shanghai). Grace Lai-Hung Wong and Vincent Wai-Sun Wong have served as

speakers and/or consultants for Echosens. Other authors have no conflicts of interest.

Abstract

Background: With metabolic dysfunction-associated fatty liver disease (MAFLD) incidence and prevalence increasing, it is necessary to identify patients with advanced fibrosis (F3-F4 stages). We evaluated the performance of new biomarkers and algorithms for diagnosing advanced fibrosis in an Asian population.

Methods: Data from two Asian cohorts (including 851 biopsy-proven MAFLD [578 from Wenzhou, 273 from Hong Kong]) were studied. The association between N-terminal propeptide of type 3 collagen (PRO-C3) and the histologic stage of liver fibrosis was analyzed by multivariable linear regression. The area under the receiver operating characteristic curve (AUROC) was used to test the diagnostic performance of serum PRO-C3 and the ADAPT score for advanced fibrosis and compared them to other established non-invasive tests.

Results: Serum PRO-C3 levels increased progressively across liver fibrosis stages and correlated with advanced fibrosis ($P<0.001$). The ADAPT score had an AUROC of 0.865 (95% confidence interval 0.829-0.901) for advanced fibrosis; the accuracy, sensitivity and negative predictive values were 81.4%, 82.2% and 96.1%, respectively. This result was better compared to that of PRO-C3 alone or other non-invasive fibrosis biomarkers (aspartate aminotransferase-to-platelet ratio index, Fibrosis-4, BARD, and NAFLD fibrosis score). In subgroup analyses (including sex, age, diabetes, NAFLD activity score, body mass index or serum alanine aminotransferase levels), the ADAPT score had good diagnostic performance.

Conclusion: PRO-C3 and the ADAPT score reliably exclude advanced fibrosis in

MAFLD patients and reduce the need for liver biopsy.

Key words:

Metabolic dysfunction-associated fatty liver disease; N-terminal propeptide of type 3 collagen; ADAPT; Fibrosis staging.

Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a highly prevalent condition worldwide and represents the most common cause of chronic liver disease in the United States, Europe and Asia [1-3]. It is estimated that the future burden of MAFLD will approximate 314 million cases by 2030 [4, 5], thereby becoming the commonest cause of cirrhosis, hepatocellular carcinoma and liver transplantation in China and other Asian countries over the coming decades [6, 7].

The newly proposed definition of MAFLD has been endorsed by various professional societies [3, 8-10], and MAFLD better identifies patients with significant fibrosis [11-13]. The severity of liver fibrosis is the strongest histologic predictor of liver-related and extra-hepatic morbidity and mortality in people with MAFLD [14-16]. In Asia, individuals with MAFLD pay little attention to their liver disease [17], so there remains a large number of undiagnosed persons with MAFLD in clinical practice [18]. Liver biopsy is the ‘gold standard’ method for staging the severity of MAFLD [19, 20], however the potential limitations of this invasive method make liver biopsy unsuitable for its wider clinical use. Thus, currently the assessment of liver fibrosis in the MAFLD population lacks an effective, accurate, and non-invasive detection tool [21, 22].

Recently, a hospital-based biopsy-confirmed MAFLD cohort from Australia showed that the N-terminal propeptide of type 3 collagen (PRO-C3) can be reliably used as a

serum marker of liver fibrosis [23, 24]. The ADAPT score (including age, diabetes, PRO-C3 and platelet count) is a derived algorithm that may accurately identify patients with significant or advanced fibrosis [24, 25]. However, before its mass adoption, this score needs to be validated in other cohorts and countries. In this large cross-sectional study, we combined two independent Asian cohorts of individuals with biopsy-proven MAFLD, and tested the diagnostic accuracy of serum PRO-C3 and the ADAPT score and compared them to other widely available non-invasive scores of advanced fibrosis.

Materials and methods

Study population

We included two Asian cohorts of adults with biopsy-proven MAFLD (i.e., Wenzhou and Hong Kong). Data from the Wenzhou cohort were collected from January 2017 to December 2020. The Hong Kong cohort included patients from a previously published study as well as additional subjects [25]. In both cohorts, MAFLD was diagnosed by the presence of hepatic steatosis on histology with at least one of the following three coexisting conditions, i.e., overweight/obesity, type 2 diabetes, or metabolic dysregulation [2, 26, 27]. All patients with MAFLD included in this study did not have hepatitis B virus or hepatitis C virus infection, excessive alcohol consumption, drug-induced liver injury, as well as known active malignancies or other causes of chronic liver disease.

Within one day of liver biopsy, fasting venous blood samples for measurement of serum liver enzymes, total bilirubin, albumin, lipids and other blood biochemical tests were obtained. Height and weight were measured for the calculation of body mass index (BMI = weight [kg]/height² [m²]). Diabetes was defined as fasting glucose level ≥ 7.0 mmol/L (≥ 126 mg/dL) or hemoglobin A_{1c} ≥ 6.5% (≥ 48 mmol/mol), a previous diagnosis of diabetes, or treatment with any anti-hyperglycemic drugs [28].

The study was approved by the institutional review boards of both centers and all participants gave written informed consent.

Pathological assessment of MAFLD

Ultrasound-guided percutaneous liver biopsy was performed using 16G or 18G needles and stained with hematoxylin-eosin and Masson trichrome. The reading and scoring of biopsy specimens were performed by an experienced pathologist in each center blinded to the clinical and biochemical data of participants. The histologic NAFLD activity score (NAS) was calculated as the sum of hepatic steatosis, lobular inflammation and hepatocyte ballooning [29-31]. The histologic stages of liver fibrosis were defined as: stage 0, no fibrosis; stage 1, peri-sinusoidal or portal venular fibrosis; stage 2, peri-sinusoidal and portal vein/periportal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis [28]. Advanced fibrosis was defined as stage F ≥ 3.

Serum PRO-C3 and ADAPT score

Type III collagen formation was assessed in serum using the PRO-C3 competitive ELISA kit from Nordic Bioscience, Herlev, Denmark, as previously described [24]. The ADAPT algorithm was calculated as follows [24]:

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

Other widely used non-invasive scores of advanced fibrosis, namely aspartate aminotransferase-platelet ratio index (APRI), fibrosis-4 (FIB-4), NAFLD fibrosis score (NFS), and BARD (BMI, aspartate aminotransferase to alanine aminotransferase ratio (AAR), diabetes) score were calculated using available clinical and laboratory variables [22, 32-34].

Statistical analysis

According to their normal or skewed distributions, continuous data were reported as means and standard deviation or medians (interquartile ranges), while categorical data were reported as a number (percentages). Univariable and multivariable linear regression analyses were performed to examine factors that significantly associated with serum PRO-C3, the ADAPT score or the other widely used non-invasive fibrosis scores. The overall diagnostic accuracy of these non-invasive scores was evaluated by receiver characteristic curve analysis and expressed as the area under the receiver operating characteristic curve (AUROC). The diagnostic accuracies of serum PRO-C3, the ADAPT score and other non-invasive fibrosis scores were determined by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), as well as positive and negative likelihood ratios (LRs). The

optimal cut-off points for each non-invasive fibrosis score were selected using the Youden's index, which attributes equal value to sensitivity and specificity. The DeLong test was performed on ROC curves by a bootstrap re-sampling method with 500 repetitions. Logistic regression analyses were performed to estimate the odds ratio (OR) and 95% confidence interval (CI). We also performed a decision curve analysis (DCA) to evaluate the new advantage, that is, whether the new model was better at identifying significant fibrosis rather than harmful (leading to unnecessary biopsy). The probability threshold reflects the diagnostic certainty of choice of liver biopsy. The curve with the greatest probability represents the best decision strategy to maximize the new advantage. All statistical analyses were performed by R version 3.6.1 (https://www.r-project.org/). Graphs were designed using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA).

Results

Patient Characteristics

We recruited 851 middle-aged Asian patients with biopsy-proven MAFLD, 578 from Wenzhou, and 273 from Hong Kong. The clinical and biochemical characteristics of the whole population are shown in **Supplemental Table 1**; the median age of patients was 43 years and 570 (67%) were men. There were 322 (38%) patients with known type 2 diabetes, 103 (12%) with advanced fibrosis (F3 or F4 stages) and approximately half of these patients had NAS $\geq$ 4.

PRO-C3 is related to the severity of liver fibrosis

We stratified patients based on the severity of liver fibrosis into two groups, F0-2 (no fibrosis to moderate fibrosis) and F3-4 stages (advanced fibrosis) (**Figure 1A**). As shown in **Table 1,** compared to those with F0-2 fibrosis, patients with advanced fibrosis had higher serum PRO-C3 levels ($p<0.001$) and ADAPT score ($p<0.001$). In addition, those with advanced fibrosis were older, more likely to be male and diabetic, and had greater adiposity measures, higher levels of serum AST, fasting glucose, and HDL-cholesterol, and lower levels of total cholesterol, LDL-cholesterol and platelets.

As shown in **Figure 1B**, serum PRO-C3 levels increased sharply across the stages of fibrosis, F0 vs. F4 ($P<0.001$), F2 vs. F3 ($P<0.001$), F1 vs. F3 ($P<0.001$) and F3 vs. F4 ($P=0.008$).

Table 2 shows the associations of serum PRO-C3 levels with clinical and biochemical parameters. Age ($\beta=0.001$, 95% CI: -0.001-0.003, $P=0.01$) and BMI ($\beta=0.008$, 95% CI: 0.003-0.013, $P<0.001$) were correlated with PRO-C3 levels; among the biochemical parameters HDL-cholesterol ($\beta=0.16$, 95% CI: 0.117-0.204, $P<0.001$) and fasting glucose ($\beta=0.012$, 95% CI: 0.001-0.023, $P=0.036$) were correlated with serum PRO-C3. The histologic parameters steatosis ($\beta=0.033$, 95% CI: 0.013-0.053, $P=0.001$), lobular inflammation ($\beta=-0.077$, 95% CI: -0.113, -0.040, $P<0.001$) and fibrosis ($\beta=0.082$, 95% CI: 0.062-0.103, $P<0.001$) were also positively correlated with PRO-C3 levels.

Diagnostic capabilities of the ADAPT score against widely used non-invasive fibrosis scores

We have tested the associations of advanced fibrosis (F3-F4) with various clinical and biochemical factors. Age (OR= 1.048, 95% CI: 1.021-1.075, P<0.001) and type 2 diabetes (OR= 2.826, 95% CI: 1.557-5.129, P<0.001) were positively associated with advanced fibrosis; other biochemical parameters, such as platelet count (OR= 0.995, 95% CI: 0.991-0.999, P=0.024) and LDL-cholesterol (OR= 0.635, 95% CI: 0.388-1.040, P=0.071) were inversely associated with advanced fibrosis (**Supplemental Table 2**). Based on the strong association between serum PRO-C3 (OR= 1.132, 95% CI: 1.092-1.174, P<0.001) and the severity of liver fibrosis, we also tested the diagnostic performance of the ADAPT score for identifying advanced fibrosis and compared it with serum PRO-C3 alone or other widely used serum-based non-invasive fibrosis scores (i.e., APRI, FIB-4, BARD and NFS).

Figure 2A shows the diagnostic performances of the different non-invasive scores for diagnosing advanced fibrosis. The AUROC of the ADAPT score was 0.865 (95% CI: 0.829-0.901) for advanced fibrosis, higher than that of PRO-C3 alone (AUROC=0.797, 95% CI: 0.753-0.842, P=0.0013) and the other widely used non-invasive fibrosis scores: 0.61 (95% CI: 0.55-0.676, P<0.001) for APRI, 0.689 (95% CI: 0.625-0.753, P<0.001) for FIB-4, 0.746 (95% CI: 0.687-0.805, P<0.001) for NFS, and 0.654 (95% CI: 0.596-0.713, P<0.001) for BARD, respectively. Compared to

312 serum PRO-C3 alone, the ADAPT score had higher sensitivity (82.2% vs. 81.6%),
313 specificity (75.8% vs. 63.7%) and concordance index (0.868, 95% CI: 0.797,0.939).
314 Similarly, the ADAPT score had higher PPV (37% vs. 23.6%). The ADAPT score
315 showed much higher performance compared to other non-invasive fibrosis scores
316 (APRI, FIB-4, NFS, and BARD) as shown in **Table 3**. Finally, the ADAPT score
317 showed similar diagnostic performance in the Wenzhou (AUROC=0.810, 95% CI:
318 0.742-0.877) and the Hong Kong (AUROC=0.809, 95% CI: 0.752-0.865) cohorts (see
319 **Supplementary Figure 1**).
320
321 The decision curve analysis (DCA) we used for assessing the ADAPT is presented in
322 **Figure 2B**. This figure analyzes the clinical utility of the ADAPT score compared
323 with APRI, FIB-4, NFS and BARD scores in identifying patients with advanced
324 fibrosis. DCA showed that from a threshold probability >10%, we could obtain more
325 net benefit by using a variance graph. In particular, if a patient's threshold probability
326 was >10% and <80%, the use of the ADAPT score for predicting the risk of advanced
327 fibrosis had more benefits than the reference strategy (using APRI, FIB-4, NFS, or
328 BARD scores).
329
330 ***Diagnostic performances of the ADAPT score and serum PRO-C3 in different***
331 ***patient subgroups***
332 We found that the ADAPT score had good performance for diagnosing advanced
333 fibrosis among different MAFLD subgroups. The AUROC of the ADAPT score was

0.841 (95% CI: 0.789-0.893) in men, and 0.889 (95% CI: 0.840-0.938) in women, respectively. Similarly, in other patient subgroups such as age < 45 years (AUROC=0.794, 95% CI: 0.716-0.873), age ≥ 45 years (AUROC=0.853, 95% CI: 0.809-0.897), NAS < 4 (AUROC=0.856, 95% CI: 0.795-0.918), NAS ≥ 4 (AUROC=0.871, 95% CI: 0.827-0.915), BMI < 25 kg/m^2 (AUROC=0.894, 95% CI: 0.816-0.972), BMI ≥ 25 kg/m^2 (AUROC=0.854, 95% CI: 0.813-0.896), ALT < 40 U/L (AUROC=0.819, 95% CI: 0.741-0.896), ALT ≥ 40 U/L (AUROC=0.886, 95% CI: 0.847-0.925), presence of diabetes (AUROC=0.806, 95% CI: 0.748-0.864) and absence of diabetes (AUROC=0.838, 95% CI: 0.766-0.910), we found comparable performances of the ADAPT score for predicting advanced fibrosis (**Figure 3**).

Circulating levels of hyaluronic acid, type III procollagen, type IV collagen, and laminin were also used as routine liver fibrosis tests in the Wenzhou biopsy cohort. **Supplemental Table 3** shows the results of univariable and multivariable logistic regression analyses preformed in the Wenzhou cohort. Interestingly, serum PRO-C3 had a better risk assessment effect on advanced fibrosis than the four aforementioned circulating parameters of liver fibrosis (adjusted OR: 1.217; 95% CI: 1.115-1.329, P<0.001).

Discussion

In this large validation study in Asian individuals, serum PRO-C3 concentration and the ADAPT score demonstrated excellent diagnostic performance for identifying

advanced fibrosis in adults with biopsy-confirmed MAFLD. The diagnostic accuracies of both tests for advanced fibrosis (F ≥ 3 stage) were significantly better than those of other widely used non-invasive scoring systems. PRO-C3 and the ADAPT algorithm reliably excluded MAFLD patients with advanced fibrosis, thereby avoiding unnecessary liver biopsies. These results suggest that serum PRO-C3 levels could be used as a routine blood biochemical indicator for liver fibrosis detection while ADAPT could further improve the accuracy of diagnosis.

The severity of liver fibrosis is the strongest prognostic factor in patients with MAFLD for adverse hepatic and extra-hepatic clinical outcomes [35]. As the prevalence of MAFLD is rapidly increasing globally, it is important to identify high-risk patient patients for specialist referral and treatment. The similar diagnostic performance of the ADAPT score in the China Wenzhou (AUROC=0.81) and China Hong Kong cohorts (AUROC=0.809) in identifying advanced fibrosis, together with prior validation in Caucasian cohorts [24], strongly supports the external validity of this score.

Through the biological processes of producing PRO-C3 during collagen production, PRO-C3 is more sensitive to the formation of active collagen fibers than static collagen accumulation [23]. Our results showed that there was no significant difference in the distribution of PRO-C3 in patients with mild to moderate fibrosis (F0-2), while in the Wenzhou cohort PRO-C3 had better performance for patients with

advanced fibrosis. In particular, we found that PRO-C3 had good diagnostic applicability even after adjustment for potentially confounding factors (the introduction of covariates in the basic model or the elimination of covariates from the complete model had an impact on the regression coefficient of PRO-C3 >10%, adjusted for matching factors including age, sex, albumin, alkaline phosphatase and fasting glucose levels). An interesting result of our study is that serum PRO-C3 remained independently associated with fibrosis stage even after adjusting for other potential confounding factors, and steatosis and lobular inflammation were independently related to PRO-C3. The use of PRO-C3 alone in the assessment of advanced fibrosis had high sensitivity (81.6%) and high NPV (96.2%). Therefore, serum PRO-C3 levels appear to be able to replace four other serum fibrosis biomarkers (such as hyaluronic acid, type III procollagen, type IV collagen, and laminin) as the best non-invasive blood marker of advanced fibrosis in patients with MAFLD, while the ADAPT score (which includes PRO-C3) has the best performance.

Our study has some important limitations that should be mentioned. Firstly, the two cohorts could be affected by bias because the results of liver biopsies may be affected by sampling variability, intra-observer and inter-observer variability. Secondly, this is a post-hoc analysis, because the samples of the two cohorts were not selected specifically for the aim of this study. Thirdly, the applicability of these data to other ethnic groups is not known. In addition, we focused on advanced fibrosis in this study

and only patients with available stored serum samples for serum PRO-C3 measurement were included in the whole cohort. Any non-invasive fibrosis score is always a trade-off between measurement accuracy (i.e., NPV and PPV) and the 'gray' areas that require further clarification with liver biopsy. The advantage of the ADAPT score is that patients with advanced fibrosis may be appropriately referred to tertiary care, while its high accuracy also means that the number of patients in the 'gray' area may increase.

In conclusion, serum PRO-C3 and the ADAPT score reliably exclude advanced fibrosis and reduce the need for liver biopsy in Asian adults with MAFLD. The ADAPT score, which requires the performance of a single biochemical test and routinely collected clinical variables, should be considered for the clinical use in primary care as a risk stratification and referral tool. Additional research to evaluate the cost-effectiveness of such a diagnostic approach is warranted.

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Figure Legends

Figure 1. (A) Comparison of baseline data between MAFLD patients with mild to moderate fibrosis (F0-2 stages) and those with advanced fibrosis (F3-4 stages). **(B)** Serum PRO-C3 levels increased in parallel with the histological severity of liver fibrosis. Each subgroup was compared using the nonparametric Kruskal–Wallis test (*P < 0.001; **P < 0.01).

Figure 2. (A) The area under the receiver operating characteristic curves (AUROC) for predicting F≥3 fibrosis. **(B)** Decision curve analysis (DCA) for the non-invasive fibrosis scores. The y-axis represents net benefits, calculated by subtracting the relative harms (true positives) from the benefits (false positives). The x-axis measures the threshold probability. A screening strategy is superior if it has the highest value compared with other models, including two simple strategies, such as all patients (sloping solid line) or no patients (horizontal solid line).

Figure 3. Forest plot for the AUROC of the ADAPT score for predicting advanced fibrosis in different patient subgroups by sex, age (years), diabetes status, serum ALT (U/L), BMI (kg/m^2), and NAFLD Activity Score (NAS).

Supplementary Figure 1. (A) The area under the receiver operating characteristic curves (AUROC) for advanced liver fibrosis in the Wenzhou cohort. **(B)** The AUROCs for advanced liver fibrosis in the Hong Kong cohort.