




# Type IV Collagen 7S Is the Most Accurate Test For Identifying Advanced Fibrosis in NAFLD With Type 2 Diabetes

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This study aimed to examine whether the diagnostic accuracy of four noninvasive tests (NITs) for detecting advanced fibrosis in nonalcoholic fatty liver disease (NAFLD) is maintained or is inferior to with or without the presence of type 2 diabetes. Overall, 874 patients with biopsy-proven NAFLD were enrolled. After propensity-score matching by age, sex, and the prevalence of dyslipidemia, 311 patients were enrolled in each group of with or without diabetes. To evaluate the effect of diabetes, we compared the diagnostic accuracy of the fibrosis-4 (FIB-4) index, the NAFLD fibrosis score (NFS), the aspartate aminotransferase to platelet ratio index (APRI), and type IV collagen 7S (COL4-7S) in patients with NAFLD with and without diabetes. The areas under the receiver operating characteristic curve (AUROC) for identifying advanced fibrosis in patients without diabetes were 0.879 for the FIB-4 index, 0.851 for the NFS, 0.862 for the APRI, and 0.883 for COL4-7S. The AUROCs in patients with diabetes were 0.790 for the FIB-4 index, 0.784 for the NFS, 0.771 for the APRI, and 0.872 for COL4-7S. The AUROC of COL4-7S was significantly larger than that of the other NITs in patients with NAFLD with diabetes than in those without diabetes. The optimal high and low cutoff points of COL4-7S were 5.9 ng/mL and 4.8 ng/mL, respectively. At the low cutoff point, the accuracy of COL4-7S was better than that of the other NITs, especially in patients with diabetes. *Conclusion:* COL4-7S measurement might be the best NIT for identifying advanced fibrosis in NAFLD, especially in NAFLD with diabetes. (*Hepatology Communications* 2021;5:559-572).

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**N**onalcoholic fatty liver disease (NAFLD) has a higher rate in developed countries than in nondeveloped countries. NAFLD has a

wide spectrum of conditions, including simple steatosis, nonalcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma.<sup>(1)</sup> Mortality of patients with NAFLD is determined by progression of liver fibrosis rather than by the grade of liver steatosis. Because the main causes of death are cardiovascular disease, cancers and liver-related disease,<sup>(2)</sup>

*Abbreviations:* ALT, alanine aminotransferase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic curve; BMI, body mass index; COL4-7S, type IV collagen 7S; DM, diabetes mellitus; FIB-4, fibrosis-4; JSG-NAFLD, Japan Study Group of NAFLD; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD Activity Score; NASH, nonalcoholic steatohepatitis; NFS, NAFLD fibrosis score; NIT, noninvasive test; PS, propensity score; ROC, receiver operating characteristic; T2DM, type 2 diabetes mellitus.

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early detection and estimation of liver fibrosis are important.<sup>(3,4)</sup>

Type 2 diabetes mellitus (T2DM), which is similar to NAFLD, is associated with metabolic syndrome. The Japan Diabetes Society reported that the leading cause of death in patients with diabetes mellitus (DM) from 2001 to 2010 in Japan was malignant neoplasm (40.7%), followed by infectious disease (17.8%), vascular disorders (13.8%), including cardiovascular disease, cerebrovascular disease and chronic kidney disease, and liver cirrhosis (3.3%).<sup>(5)</sup> In this report, mortality related to liver disease, such as liver cirrhosis and hepatocellular carcinoma, was estimated to be approximately 10%, and the importance of identifying advanced fibrosis in patients with T2DM was emphasized. In patients with T2DM, the prevalence of advanced fibrosis in those with NAFLD is 7%-12%.<sup>(6-8)</sup>

NAFLD and DM affect each other. As these conditions progress, managing treatment becomes difficult. NAFLD should be recognized as one of the final organ disorders of DM. The prevalence of NAFLD is often affected by an increase in obesity, and this is thought to be one phenotype of metabolic syndrome, although there are patients with NAFLD who are not obese and taken to metabolic syndrome. NAFLD is a complicated disease, and the relation of NAFLD and DM is still unclear. Therefore, making a diagnosis and treatment for liver disease and DM are difficult for clinicians.<sup>(9,10)</sup>

In a previous study, diabetes was significantly associated with incident NAFLD and hepatocellular carcinoma. Interventions that improve metabolic abnormalities in patients with diabetes (weight loss, glycemic control, and treatment with specific drugs for hyperglycemia or dyslipidemia) are also beneficial for fatty liver disease.<sup>(11)</sup> An effective treatment has not been established for NAFLD, and kinesitherapy and improvement of lifestyle are currently the only treatments. However, examination of an effective treatment on the basis of the pathophysiological relationship of DM and NAFLD is necessary in the future.<sup>(12)</sup>

To diagnose the stage of fibrosis in NAFLD, percutaneous liver biopsy is still the gold standard. However, disadvantages of this technique include the risk of complications because of invasiveness of the procedure, sampling errors, and high cost.<sup>(13)</sup> Therefore, there is considerable interest in using validated noninvasive tests (NITs) of fibrosis in clinical practice. Of these scoring systems, the fibrosis-4 (FIB-4) index and NAFLD fibrosis score (NFS) are the most useful for excluding advanced fibrosis (brunt stage; F = 3, 4) in patients with NAFLD.<sup>(14)</sup> The area under the receiver operating characteristic curve (AUROC) for predicting advanced fibrosis in NAFLD using the FIB-4 index and NFS is 0.86 and 0.88, respectively. The American Association for the Study of Liver Diseases (AASLD) created a practice guidance for NAFLD in 2018.<sup>(15)</sup> In this guideline,

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the NFS and FIB-4 index are clinically more useful NITs for identifying patients with NAFLD with a higher likelihood of having bridging fibrosis (stage 3) or cirrhosis (stage 4) than other scores, such as the body mass index (BMI), aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio, and diabetes mellitus (BARD) score, the AST to platelet ratio index (APRI), and the AST/ALT ratio. As previously reported, the utility of some noninvasive scoring systems (BAAT [BMI, age, alt, and triglycerides] score, BARD score, and NFS) was limited in a population with T2DM by inclusion of age, BMI, and high levels of indeterminate results.<sup>(15)</sup>

In this study, we compared four NITs for detecting advanced fibrosis of NAFLD in a clinical population with biopsy-proven NAFLD. We evaluated the diagnostic accuracy of these NITs in patients with NAFLD and T2DM.

## Patients and Methods

We performed a cross-sectional study to estimate the accuracy of NITs in patients with NAFLD and T2DM. In this study, 874 patients were enrolled in the Japan Study Group of NAFLD (JSG-NAFLD), and all patients underwent a liver biopsy from 2002 to 2015. The JSG-NAFLD included the following nine Japanese hepatology centers: Kyoto Prefectural University of Medicine, Nara City Hospital, Yokohama City University Graduate School of Medicine, Hiroshima University, Kochi Medical School, Saga University, Osaka City University, Asahikawa Medical College, and Saiseikai Suita Hospital.

All patients were diagnosed with the presence or absence of T2DM in accordance with the guideline of the Japan Society of Diabetes as follows: patients who had a diagnosis of diabetes; patients with documented use of oral hypoglycemic medication, whose random glucose levels exceeded 200 mg/dL; or patients with fasting plasma glucose levels >126 mg/dL and hemoglobin A1c values  $\geq$  6.6%.<sup>(16)</sup>

The diagnosis of NAFLD was based on previously reported criteria<sup>(17)</sup> and appropriately excluded liver disease with clear etiologies, such as viral and autoimmune hepatitis, and alcoholic liver disease. Written, informed consent was obtained from all patients at the time of their liver biopsy. This study was approved by the institutional review boards of all participating

institutions, and it conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

## COVARIATES

Physical characteristics and clinical laboratory data were examined for each patient. Physical factors included age, sex, and BMI, and clinical characteristics included a confirmed diagnosis of hypertension, diabetes, and dyslipidemia. Blood samples were taken in the morning after a 12-hour overnight fast, and clinical laboratory data were collected. These parameters were measured using standard techniques in clinical laboratories. Type IV collagen 7S (COL4-7S) was measured by double antibody radioimmunoassay. The cutoff point was set two cutoff points, like other NITs, so that sensitivity and specificity for advanced fibrosis were at least 90% with using the overall population. BMI was calculated as weight in kilograms divided by height in square meters. Obesity was defined as BMI > 25.0 kg/m<sup>2</sup> according to the criteria of the Japan Society for the Study of Obesity.<sup>(18)</sup> Dyslipidemia was diagnosed if cholesterol levels were >220 mg/dL, low-density lipoprotein cholesterol levels were >140 mg/dL, high-density lipoprotein cholesterol levels were <40 mg/dL, and/or triglyceride levels were >160 mg/dL. Hypertension was diagnosed if the patient was on antihypertensive medication and/or had a resting recumbent blood pressure  $\geq$ 140/90 mm Hg on at least two occasions.

## LIVER HISTOLOGY AND DIAGNOSIS OF NAFLD AND NASH

All patients underwent percutaneous liver biopsy under ultrasonic guidance. Liver specimens were embedded in paraffin and stained with hematoxylin and eosin, Masson's trichrome, and reticulin silver stain. Two pathologists and one hepatologist (S.I., K.S, and Y.S.), who were blinded to all clinical and identifying data, reviewed the liver biopsy specimens. An adequate liver biopsy sample was defined as a biopsy specimen with a length greater than 1.5 cm and/or more than six portal tracts. NASH was defined as steatosis with lobular inflammation and ballooning degeneration with or without Mallory-Denk bodies or fibrosis. Patients whose liver biopsy specimens showed steatosis or steatosis with nonspecific inflammation were identified as the NAFL cohort. The severity of hepatic fibrosis

(stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis.<sup>(17)</sup> Advanced fibrosis was defined as having a severity greater than stage 3. Scoring of steatosis included microvesicular and macrovesicular steatosis, and was based on the percentage area of the parenchyma that was fatty as follows: <33% was considered as mild, 33%–65% as moderate, and >66% as advanced. The activity of NAFLD was estimated by the NAFLD Activity Score (NAS), as proposed by Kleiner et al.,<sup>(19)</sup> which was the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2).

## NITs FOR PREDICTING HEPATIC FIBROSIS

The FIB-4 index, the NFS, the APRI, and COL4-7S were assessed in each patient. The FIB-4 index was calculated using the following formula: (age [years] × AST/platelet count [ $\times 10^9/L$ ] × ALT<sup>1/2</sup>).<sup>(20)</sup> The NFS was calculated using the following formula:  $-1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m}^2) + 1.13 \times \text{impaired glucose tolerance/DM (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} \times 0.013 \times \text{platelet count (}\times 10^9/L) - 0.66 \times \text{albumin (g/dL)}$ .<sup>(21)</sup> The APRI was calculated using the following formula:  $([\text{AST/upper limit of normal}]/\text{platelet count } [\times 10^9/L] \times 100 [\times \text{upper limit of normal, AST upper level of normal, or } 56 \text{ IU/L}])$ .<sup>(22)</sup> According to accumulating evidence, the high cutoff points were 2.67 for the FIB-4 index, 0.675 for the NFS, and 2.0 for the APRI, and the low cutoff points were 1.30 for the FIB-4 index,  $-1.45$  for the NFS, and 0.5 for the APRI.<sup>(21–23)</sup>

## STATISTICAL ANALYSIS

Data are expressed as the median and range for quantitative data or as number and percentage for qualitative data. Statistical differences between two or three groups were analyzed using the Mann-Whitney U test and Kruskal-Wallis analysis for quantitative data, and using Fisher's test or the chi-square test for qualitative data. Normality was confirmed using Shapiro-Wilk analysis. The propensity score (PS) model was estimated using a logistic regression model that was adjusted for the patients' characteristics listed

in Table 1, as well as for age, sex, and the prevalence of dyslipidemia, because these variables were prognostically significant in other studies.<sup>(24)</sup> Possible confounders were chosen for their potential association with the outcome of interest on the basis of clinical knowledge. One patient with NAFLD and T2DM was matched to many patients with NAFLD without T2DM using nearest-neighbor matching without replacement. The PSs were matched using a caliper width of 1/5 of the logit of the SD. After PS matching, receiver operating characteristic (ROC) curve analysis was performed for each NIT to identify advanced fibrosis in NAFLD. Furthermore, each AUROC was compared with the DeLong test for the presence or absence of DM.

All analyses were performed using JMP 12 (SAS Institute Inc., Cary, NC) and R statistics version 3.4.0. Nominal, two-sided *P*-values were used and were considered statistically significant for values <0.05 *a priori*.

## Results

### CHARACTERISTICS OF PATIENTS WITH NAFLD AND T2DM AFTER PS MATCHING WITH AGE, SEX, AND PREVALENCE OF DYSLIPIDEMIA

A total of 874 patients with NAFLD were included in this study. Of them, 497 (43.1%) patients had T2DM (Table 1). Age, the rate of female sex, and the prevalence of dyslipidemia, hypertension, NASH, and advanced liver fibrosis were significantly higher in patients with NAFLD with T2DM than in those without T2DM. Furthermore, NIT results, including COL4-7S, the FIB-4 index, the NFS, and the APRI, were significantly higher in patients with NAFLD with T2DM compared to those without T2DM.

PS matching was performed to adjust for each group with NAFLD with or without T2DM by age, sex, and the prevalence of dyslipidemia. Finally, 311 patients were enrolled in each group of patients with NAFLD with and without T2DM after PS matching. The characteristics of patients with NAFLD and T2DM were a higher rate of obesity, lower albumin levels, higher prevalence of hypertension, and higher rate of advanced liver fibrosis than those without T2DM.

TABLE 1. CHARACTERISTICS OF PATIENTS WITH NAFLD WITH OR WITHOUT T2DM

Variables	Diabetes Absence (n = 497)	Diabetes Presence (n = 377)	P Value
Age (years)	51 (14-84)	60 (17-92)	<0.001
Gender (female)	207 of 497 (41.6%)	214 of 377 (56.8%)	<0.001
BMI (kg/m <sup>2</sup> )	27.0 (10.3-49.5)	27.3 (16.5-44)	0.752
AST (IU/L)	44 (16-331)	43 (15-202)	0.993
ALT (IU/L)	73 (16-547)	60 (11-358)	0.003
Albumin (mg/dL)	4.5 (3.1-5.8)	4.3 (2.8-5.4)	<0.001
Platelets ( $\times 10^9$ /UL)	22.8 (6.8-75.1)	21.1 (4.6-78.5)	<0.001
T-Bil (mg/dL)	0.66 (0.30-3.59)	0.74 (0.25-2.31)	0.396
TG (mg/dL)	152 (43-659)	147 (49-920)	0.489
Total cholesterol (mg/dL)	206 (77-436)	207 (77-372)	0.726
HDL-C (mg/dL)	48 (23-290)	49 (22-99)	0.115
LDL-C (mg/dL)	129 (44-246)	126 (8-294)	0.668
NASH (yes)	320 of 497 (64.4%)	279 of 378 (73.8%)	0.003
Dyslipidemia (yes)	345 of 490 (70.4%)	296 of 374 (79.1%)	0.004
Hypertension (yes)	106 of 413 (25.7%)	152 of 318 (47.8%)	<0.001
COL4-7S (ng/mL)	4.1 (0.2-13)	4.5 (1.0-55.5)	<0.001
FIB-4 index	1.09 (0.17-7.51)	1.60 (0.24-10.74)	<0.001
NFS	-2.07 (-9.08 to 2.15)	-0.56 (-8.77 to 2.83)	<0.001
APRI	0.67 (0.13-4.78)	0.72 (0.15-4.42)	0.046
Brunt grade (1/2/3)	197/211/82 (40.2%/43.1%/16.7%)	139/145/58 (40.6%/42.4%/17.0%)	—
Brunt stage (0/1/2/3/4)	222/170/66/25/16 (44.5%/34.1%/13.2%/5.0%/3.2%)	129/112/67/47/23 (34.1%/29.6%/17.7%/12.4%/6.1%)	—
Advanced fibrosis (F3 and F4)	41 of 499 (8.2%)	70 of 378 (18.5%)	<0.001
NAS total (1/2/3/4/5/6/7/8)	23/39/57/113/91/61/28/1 (5.6%/9.4%/13.8%/27.4%/22.0%/14.8%/6.8%/0.2%)	3/13/40/37/60/52/18/1 (1.3%/5.4%/17.9%/18.3%/25.4%/21.9%/9.4%/0.4%)	—
Steatosis (0/1/2/3)	2/193/201/88 (0.4%/39.9%/41.5%/18.2%)	0/114/115/49 (40.7%/42.5%/16.8%)	—
Inflammation (0/1/2/3)	76/222/97/18 (18.4%/53.8%/23.5%/4.4%)	26/111/69/18 (11.6%/49.6%/30.8%/8.0%)	—
Ballooning (0/1/2)	78/146/189 (18.9%/35.3%/45.8%)	23/72/129 (10.3%/32.1%/57.6%)	—

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; T-Bil, total bilirubin; TG, triglycerides.

There was no significant difference in the prevalence of liver cirrhosis between the groups. COL4-7S levels and the NFS were significantly higher in patients with NAFLD with T2DM than the levels in those without T2DM, whereas the FIB-4 index and APRI were not different between the two groups (Table 2). The distribution of these NITs in the presence or absence of T2DM after PS matching is shown in Fig. 1. In NAFLD with DM and NAFLD without DM, there were significantly equilateral correlations between these NITs and NASs histologically in the total NAS, inflammation, and ballooning (Supporting Table S1).

## ROC CURVE ANALYSIS

ROC curves were developed to estimate the utility of each NIT in predicting advanced fibrosis (stages

3 and 4) with or without T2DM (Fig. 2A). The AUROCs in the group of patients without T2DM were 0.879 for the FIB-4 index, 0.851 for the NFS, 0.862 for the APRI, and 0.883 for COL4-7S. The AUROCs in the group of patients with T2DM were 0.790 for the FIB-4 index, 0.784 for the NFS, 0.771 for the APRI, and 0.872 for COL4-7S. The AUROCs of the FIB-4 index ( $P = 0.038$ ) and APRI ( $P = 0.026$ ) in patients with T2DM were significantly smaller than those in patients without T2DM. Additionally, the AUROC of the NFS in patients with T2DM tended to be smaller than that in patients without T2DM ( $P = 0.159$ ). There was no significant difference in the AUROC of COL4-7S between patients with and without T2DM.

To compare the AUROC of COL4-7S with that of other fibrosis scores in patients with or without T2DM (Fig. 2B), the change in the AUROC

**TABLE 2. CHARACTERISTICS OF PATIENTS WITH NAFLD AFTER PS MATCHING BY AGE, SEX, AND PRESENCE OF DYSLIPIDEMIA**

Clinical Parameters	Diabetes Absence (n = 311)	Diabetes Presence (n = 311)	PValue
Age (years)	58 (16-84)	58 (17-83)	0.9704
Gender (female)	168 of 311 (54.0%)	164 of 311 (52.7%)	0.8095
BMI (kg/m <sup>2</sup> )	26.7 (17.7-43.5)	27.4 (17.3-44.0)	0.0369
AST (IU/L)	44 (16-208)	43 (15-202)	0.7037
ALT (IU/L)	66 (16-298)	64 (12-358)	0.7683
Albumin (mg/dl)	4.4 (3.1-5.7)	4.3 (2.8-5.4)	0.0219
Platelets ( $\times 10^9$ /uL)	22 (6.8-75.1)	21.4 (4.6-78.5)	0.2182
T-Bil (mg/dL)	0.66 (0.36-3.59)	0.79 (0.37-2.31)	0.2893
GGT (IU/L)	60 (14-534)	63 (12-1464)	0.065
Total cholesterol (mg/dL)	208 (77-335)	207 (77-372)	0.351
TG (mg/dL)	149 (44-642)	150 (49-920)	0.9632
HDL-C (mg/dL)	51 (23-290)	49 (22-99)	0.2185
LDL-C (mg/dL)	131 (44-246)	125 (8-294)	0.3082
HbA1c (%)	5.8 (4.5-6.5)	6.7 (5.1-12.8)	<0.001
Dyslipidemia (yes)	240 of 311 (77.2%)	242 of 311 (77.8%)	0.9235
Hypertension (yes)	86 of 266 (32.3%)	126 of 271 (46.5%)	<0.001
COL4-7S (ng/mL)	4.2 (2.5-13)	4.4 (1-55.5)	0.0032
FIB-4 index	1.33 (0.17-7.51)	1.53 (0.24-9.32)	0.1638
NFS	-1.847 (-9.081 to -2.147)	-0.743 (-8.764 to 3.250)	<0.001
APRI	0.694 (0.126-4.277)	0.725 (0.149-4.420)	0.3891
Brunt grade (1/2/3)	137/118/51 (44.8%/38.6%/16.6%)	120/115/48 (42.4%/40.6%/17.0%)	—
Brunt stage (0/1/2/3/4)	128/104/44/23/12 (41.1%/33.4%/14.1%/7.4%/3.9%)	106/96/55/39/15 (34.1%/30.9%/17.7%/12.5%/4.8%)	—
Advanced fibrosis (F3 and F4)	35 of 311 (11.3%)	54 of 311 (17.4%)	0.0388
Liver cirrhosis (F4)	12 of 311 (3.9%)	15 of 311 (4.8%)	0.695
NAS total (1/2/3/4/5/6/7/8)	12/19/34/66/60/42/18/1 (4.8%/7.5%/13.5%/26.2%/23.8%/16.7%/7.1%/0.4%)	3/11/33/31/49/43/12/1 (1.6%/6.0%/18.0%/16.9%/26.8%/23.5%/6.6%/0.5%)	—
Steatosis (0/1/2/3)	135/117/52 (44.4%/38.5%/17.1%)	120/115/48 (42.4%/40.6%/17.0%)	—
Inflammation (0/1/2/3)	43/132/62/15 (17.0%/52.4%/24.6%/6.0%)	24/91/56/12 (13.1%/49.7%/30.6%/6.6%)	—
Ballooning (0/1/2)	34/87/131 (13.5%/34.5%/52.0%)	21/61/101 (11.5%/33.3%/55.2%)	—

Note: Caliper: 0.20.

Abbreviations: HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; T-Bil, total bilirubin; TG: triglycerides.

was tested using the DeLong test. In NAFLD with T2DM, the AUROC of COL4-7S was significantly higher than those of other NITs. However, there were no significant differences between AUROCs of COL4-7S and the other NITs in NAFLD without T2DM.

To estimate the difference in the AUROC between patients with NAFLD with T2DM and those without T2DM, we examined the clinical background of these patients with advanced fibrosis or without fibrosis (Table 3). In patients without T2DM, age, AST levels, and COL4-7S levels were significantly higher and the platelet count was significantly lower

in patients with stages 3 and 4 than in those with stages 0-2. In patients with T2DM, similarly, age, BMI, AST levels, and COL4-7S levels were significantly higher and the platelet count was significantly lower in stages 3 and 4 than in stages 0-2. Results of NITs, such as the FIB-4 index, NFS, and APRI, were significantly higher in stages 3 and 4 than in stages 0-2 in patients with and those without T2DM. In patients with stages 3 and 4, the platelet count tended to be higher and age tended to be lower in the advanced fibrosis group with T2DM (n = 54) than in the advanced fibrosis group without T2DM (n = 35).

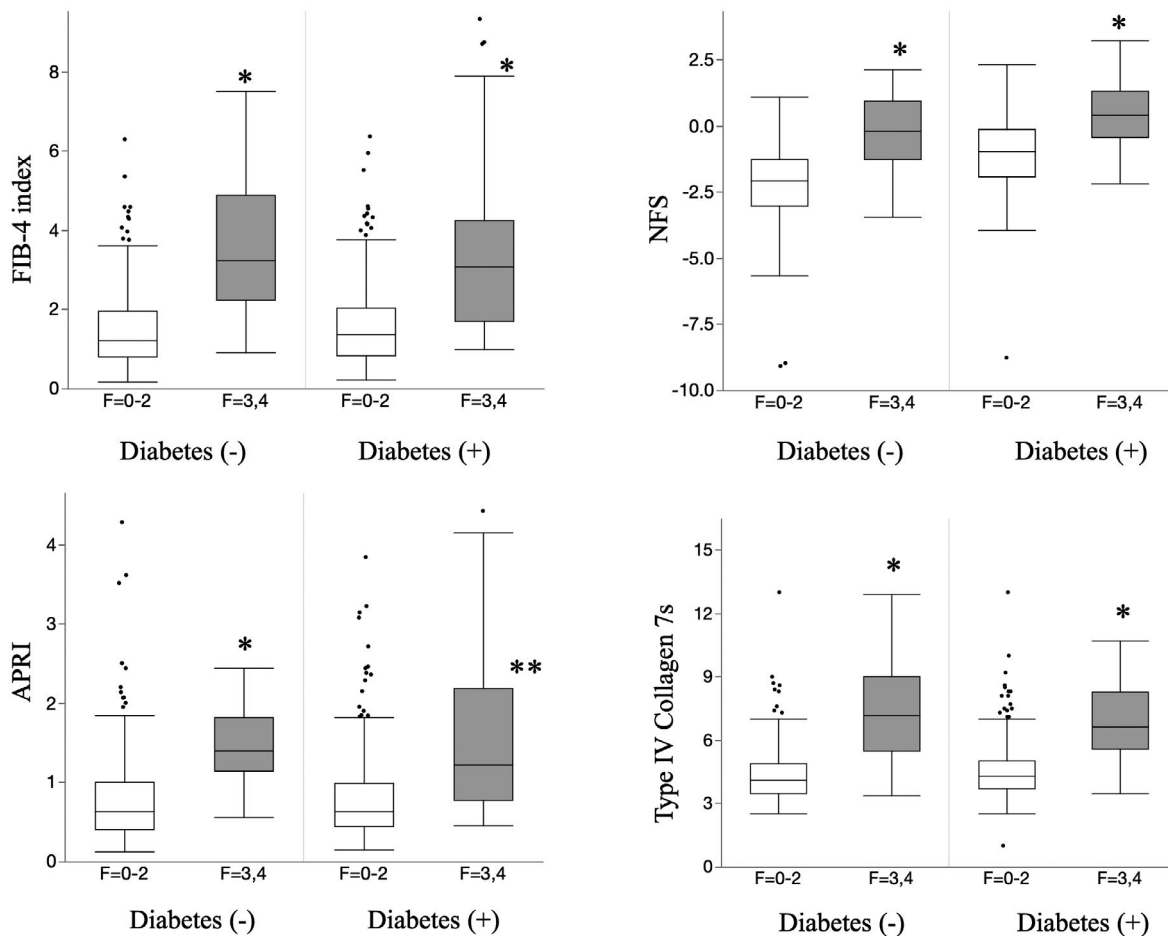


FIG. 1. Box plot of NITs for advanced fibrosis of NAFLD with or without T2DM. \* $P < 0.01$  versus F = 0-2.

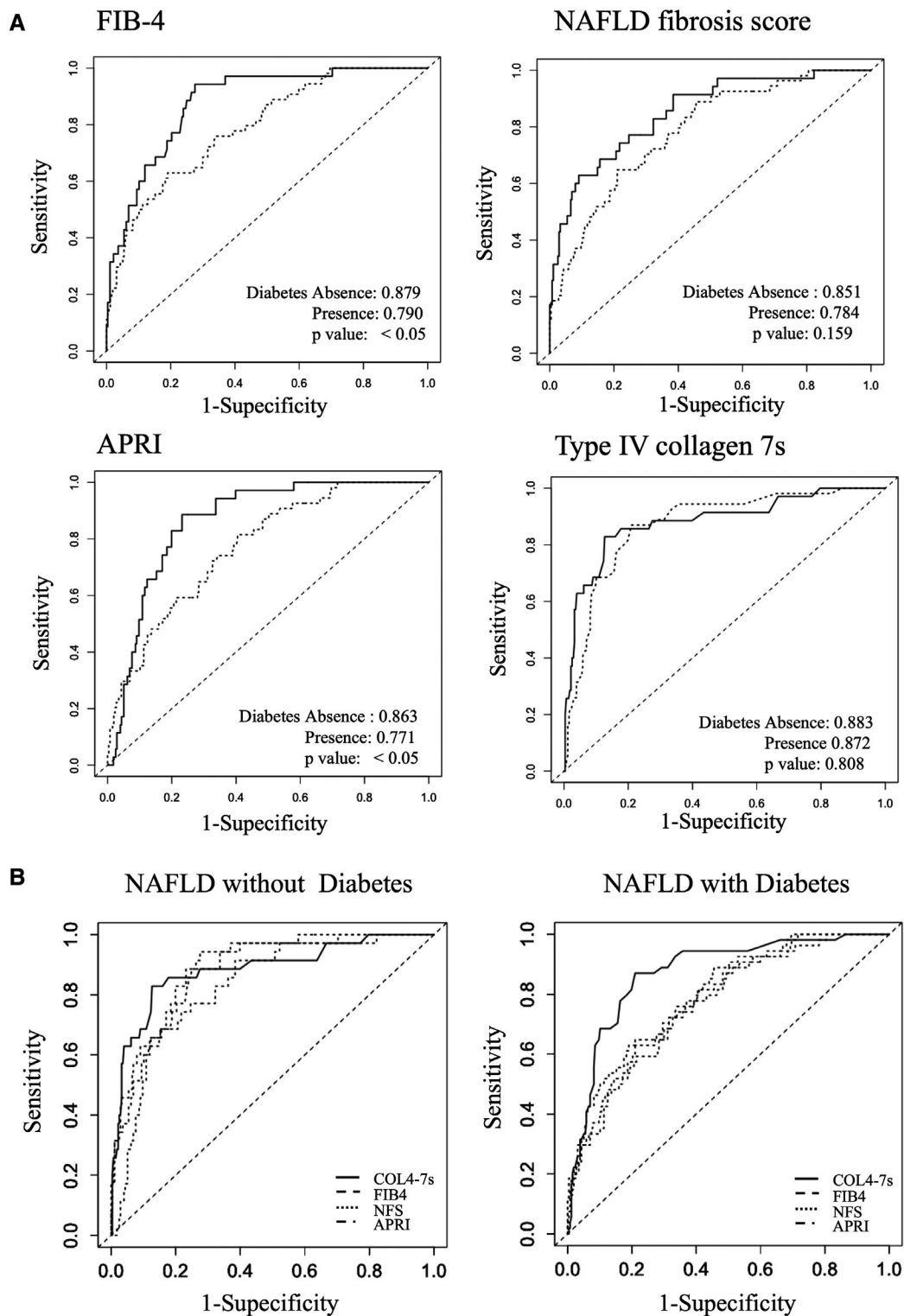
## CLINICAL UTILITY OF NITs FOR PREDICTING ADVANCED FIBROSIS

Using the overall population, two cutoff points of COL4-7S were selected, so that sensitivity and specificity for advanced fibrosis were at least 90%. The high cutoff point was 5.9 ng/mL and the low cutoff point was 4.8 ng/mL for COL4-7S. Low and high cutoff points of each NIT are shown in Fig. 3. We compared the clinical utility of these NITs for identifying advanced fibrosis in patients with T2DM when the proposed cutoff point of each NIT was applied (Table 4). Using the high cutoff points, sensitivity, the false negative rate, and the negative predictive value were best for COL4-7S among the NITs in both groups, regardless of the presence of T2DM. In NAFLD with T2DM, sensitivity and the negative

predictive value for advanced fibrosis of COL4-7S were better than those of the other NITs. The accuracy of COL4-7S did not differ from that of other NITs. In the low cutoff points, specificity, the false positive rate, and the positive predictive value of COL4-7S were better than those of the other NITs. The accuracy of COL4-7S was superior to that of the other NITs, especially in patients with NAFLD with T2D.

## CORRELATIONS BETWEEN AGE AND PLATELET COUNT OR COL4-7S

We investigated the relation between age and platelet count or COL4-7S (Fig. 4). There was a significant negative correlation between age and platelet count in patients with NAFLD with T2DM (correlation

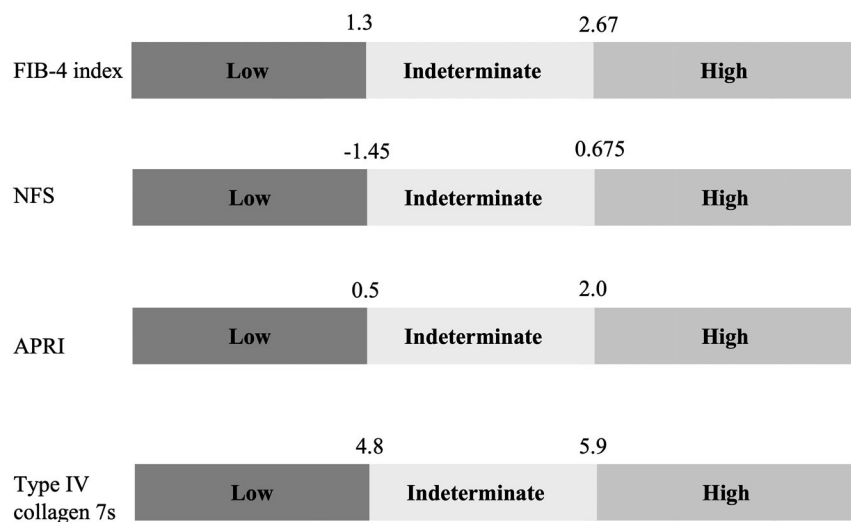


**FIG. 2.** (A) ROC curve analysis of the FIB-4 index, NFS, APRI, and COL4-7S. The solid line indicates the absence of T2DM, and the broken line indicates the presence of T2DM. (B) DeLong test of each NIT of NAFLD with or without T2DM.



**TABLE 3. COMPARISON BETWEEN FACTORS OF NONINVASIVE FIBROSIS MARKERS FOR DIAGNOSING ADVANCED FIBROSIS IN PATIENTS WITH NAFLD, WITH OR WITHOUT T2DM**

Clinical Parameters	Diabetes Absence		PValue	Diabetes Presence		PValue
	F0-F2 (n = 276)	F3 and F4 (n = 35)		F0-F2 (n = 257)	F3 and F4 (n = 54)	
Age (years)	56 (16-84)	65 (42-79)	<0.01	57 (17-82)	60 (45-83)	0.035
BMI (kg/m <sup>2</sup> )	26.7 (17.7-43.5)	26.9 (20.4-39.0)	0.621	27.2 (17.3-44.0)	28.6 (19.6-40.1)	0.018
Albumin (mg/dL)	4.5 (3.2-5.7)	4.1 (3.1-4.8)	<0.01	4.4 (2.8-5.4)	4.2 (3.1-4.9)	<0.01
AST (IU/L)	42 (16-208)	55 (36-119)	<0.01	41 (15-202)	58 (27-186)	<0.01
ALT (IU/L)	67 (16-298)	62 (25-227)	0.826	64 (12-358)	62 (17-218)	0.520
Platelets ( $\times 10^9$ /uL)	22.7 (10.5-75.1)	14.6 (6.8-28.7)	<0.01	22.2 (8.2-78.5)	17.6 (4.6-31.1)	<0.01
FIB-4 index	1.23 (0.17-6.29)	3.24 (0.91-7.51)	<0.01	1.37 (0.24-6.36)	3.08 (1.00- 9.32)	<0.01
NFS	-2.08 (-9.08 to 1.10)	-0.21 (-3.45 to 2.15)	<0.01	-0.97 (-8.76 to 2.34)	0.43 (-2.19 to 3.25)	<0.01
APRI	0.64 (0.13-4.28)	1.41 (0.56-2.45)	<0.01	0.64 (0.15-3.84)	1.23 (0.46-4.42)	<0.01
COL4-7S (ng/mL)	4.1 (2.5-13)	7.2 (3.4-12.9)	<0.01	4.3 (1-55.5)	6.7 (3.5-23)	<0.01

**FIG. 3.** Low and high cutoff points of each NIT applied in this study.

coefficient:  $-0.4305$ ,  $P < 0.001$ ) and in those without T2DM (correlation coefficient:  $-0.4069$ ,  $P < 0.001$ ). However, there was a significant positive correlation between age and COL4-7S in patients with NAFLD with T2DM (correlation coefficient:  $0.1977$ ,  $P < 0.001$ ) and in those without T2DM (correlation coefficient:  $0.3055$ ,  $P < 0.001$ ).

## Discussion

In this study, we showed that COL4-7S measurement was significantly better than conventional NITs for excluding advanced fibrosis of NAFLD

with T2DM. Furthermore, COL4-7S was similar to other NITs in NAFLD without T2DM. In this study, we compared the diagnostic accuracy of NITs, including the FIB-4 index, the NFS, the APRI, and COL4-7S, for detecting advanced fibrosis between patients with NAFLD and T2DM and those without T2DM. In patients without T2DM, each test was similar for diagnosing advanced fibrosis in NAFLD. However, in patients with T2DM, the AUROCs of the NITs of the FIB-4 index, NFS, and APRI were smaller than 0.80 (Fig. 2A). The AUROC of COL4-7S was 0.876 in patients with NAFLD and T2DM. The diagnostic accuracy of NITs other than COL4-7S in patients with

**TABLE 4. COMPARISON OF THE CLINICAL UTILITY OF EACH NIT AT HIGH AND LOW CUTOFF POINTS FOR ADVANCED FIBROSIS IN NAFLD**

High cutoff point	NIT	Sensitivity	Specificity	FP	FN	PPV	NPV	Accuracy
Diabetes absence	FIB-4 index > 2.67	0.657	0.877	0.123	0.343	0.404	0.953	0.852
	NFS > 0.675	0.286	0.989	0.011	0.714	0.769	0.916	0.913
	APRI > 2.0	0.114	0.964	0.036	0.886	0.286	0.896	0.865
	COL4-7S > 5.9	0.686	0.899	0.101	0.314	0.462	0.958	0.875
Diabetes presence	FIB-4 index > 2.67	0.537	0.856	0.144	0.463	0.439	0.898	0.797
	NFS > 0.675	0.426	0.891	0.109	0.574	0.451	0.881	0.813
	APRI > 2.0	0.278	0.957	0.043	0.722	0.577	0.863	0.842
	COL4-7S > 5.9	0.685	0.872	0.128	0.315	0.529	0.929	0.839
<i>Low cutoff point</i>								
Diabetes absence	FIB-4 index > 1.30	0.971	0.547	0.453	0.029	0.214	0.993	0.595
	NFS > -1.45	0.771	0.685	0.315	0.229	0.237	0.959	0.695
	APRI > 0.5	1.00	0.362	0.638	0	0.166	1.000	0.434
	COL4-7S > 4.8	0.886	0.725	0.275	0.114	0.290	0.980	0.743
Diabetes presence	FIB-4 index > 1.30	0.889	0.475	0.525	0.111	0.262	0.953	0.547
	NFS > -1.45	0.926	0.358	0.642	0.074	0.233	0.958	0.457
	APRI > 0.5	0.944	0.327	0.673	0.056	0.228	0.966	0.434
	COL4-7S > 4.8	0.889	0.689	0.311	0.111	0.375	0.967	0.723

Abbreviations: FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value.

NAFLD with T2DM was lower than that in those without T2DM. However, the diagnostic accuracy of COL4-7S in patients with NAFLD with T2DM was similar to that in those without T2DM. COL4-7S is considered useful for excluding advanced fibrosis of NAFLD, especially with the presence of T2DM. The diagnostic accuracy of the FIB-4 index and NFS for advanced fibrosis fluctuates by age, and resetting the cutoff point by each age group is necessary.<sup>(25,26)</sup> In our study, we matched NAFLD with and without T2DM by age, sex, and the prevalence of dyslipidemia, and found that these variables were significantly different between the two groups.

COL4-7S is a fragment of collagen type IV and is part of the extracellular matrix, which forms the basement membrane. COL4-7S is an established biochemical marker of liver fibrosis.<sup>(27,28)</sup> An increase in serum COL4-7S levels is accompanied by progression of liver fibrosis in various chronic types of hepatitis, including NASH.<sup>(28)</sup> Additionally, the efficacy for diagnosis of advanced fibrosis in NASH has been established.<sup>(29,30)</sup> A previous study showed the efficacy of COL4-7S for diagnosing NASH or advanced fibrosis of NAFLD.<sup>(30)</sup> Sumida et al. reported that the NAFIC score, which consists of ferritin, insulin, and COL4-7S, predicted NASH.<sup>(29)</sup> These authors found that the AUROC for predicting NASH was 0.851

in the estimation group and 0.782 in the validation group. Okanou et al. reported that the CA index, which consists of COL4-7S and AST, predicted NASH or NASH-related fibrosis.<sup>(31)</sup> The AUROC of the CA-fibrosis index for predicting NASH-related fibrosis was 0.918. Therefore, COL4-7S might be an important parameter for diagnosing NASH or advanced fibrosis in NAFLD.

The FIB-4 index and NFS are recommended for diagnosing advanced liver fibrosis of NAFLD primarily according to the guideline of the AASLD and the European Association for the Study of the Liver.<sup>(12,32)</sup> However, our study showed that the diagnostic accuracy of the FIB-4 index and NFS was inferior to that of COL4-7S in NAFLD with T2DM. The reason why these conventional NITs are inferior, especially in patients with T2DM, remains to be resolved. One reason for our finding is that the FIB-4 index and NFS include age. In the advanced stage (stages 3 and 4) in our study, patients with T2DM tended to younger (60 [45-83] years) compared to those without T2DM (65 [42-79] years) (Table 3). Cross-sectional data from a multicenter study by the JSG-NAFLD showed that the presence of T2DM was strongly associated with the severity of liver fibrosis in Japanese patients with NASH.<sup>(33)</sup> Adams et al. reported that T2DM and a low initial stage of fibrosis were associated with a

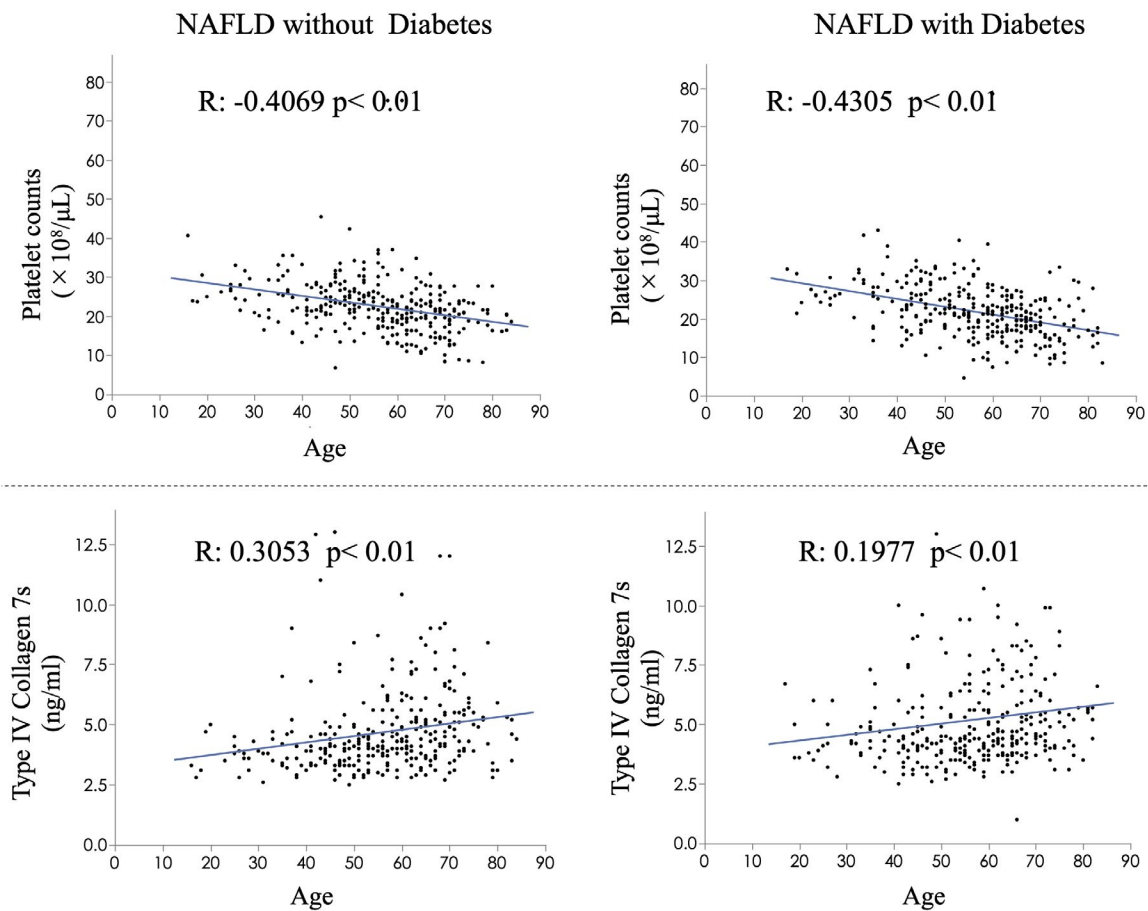


FIG. 4. Scatter plot of the correlation between age and platelet count or COL4-7S, with or without T2DM.

higher rate of progression of fibrosis in 103 patients with NAFLD who underwent serial liver biopsies.<sup>(34)</sup> They showed a mean interval of  $3.2 \pm 3.0$  years between biopsies. In another cohort of Japanese patients with NAFLD, the presence of T2DM was an independent risk factor for progression to advanced liver fibrosis (hazard ratio, 1.879), as evaluated by the FIB-4 index in addition to advanced age and low albumin concentrations. Furthermore, a longer duration of T2DM is an independent risk factor for progressive fibrosis in patients with NAFLD.<sup>(35)</sup> The reason why patients with T2DM are younger in the advanced stage may be explained by T2DM being a significant risk factor for rapid progression of hepatic fibrosis in NAFLD.<sup>(36)</sup> Another reason for our finding is that conventional NITs, such as the FIB-4 index, NFS and APRI, are affected by the platelet count, because their formulas include the platelet count. In the advanced stage (stages 3 and 4) in our study, the platelet count

appeared to be higher in patients with T2DM compared to those without T2DM, but this was not significant (Table 3). The reason for this lack of finding remains unknown. We found that the platelet count was negatively correlated with age in patients with T2DM and in those without T2DM (Fig. 4). The platelet count is decreased in parallel with aging. In the advanced stage, patients with T2DM might have shown a higher platelet count than those without T2DM, because patients with T2DM were younger. Another explanation for our finding is that the platelet count is elevated in patients with metabolic syndrome.<sup>(37)</sup> Jesri et al. reported that the platelet count increased in parallel with obesity, insulin resistance, and physical inactivity. Obesity, insulin resistance, and diabetes are associated with higher levels of several inflammatory markers, including interleukin-6. Elevated interleukin-6 levels may be responsible for increased platelets in NAFLD with T2DM.<sup>(38-41)</sup>

The FIB-4 index and NFS are useful NITs for diagnosing advanced fibrosis in NAFLD without T2DM. However, there must be caution in diagnosing advanced fibrosis of NAFLD. This is because some studies have noted the problem that diagnostic accuracy of these tests decreases with increasing age of patients. Therefore, our research group and others have proposed adaptive cutoff points for the age of patients.<sup>(25,26)</sup> Researchers must be aware that these NITs are affected by age. Similar to our study, previous studies have reported that the AUROC tends to decrease in NAFLD with T2DM compared to NAFLD without T2DM for diagnosis of advanced fibrosis  $\geq$  stage 3 and significantly decreases for diagnosis of cirrhosis.<sup>(42)</sup> The FIB-4 index, NFS, and APRI are useful diagnostic methods, but in the diagnosis of advanced fibrosis of NAFLD, age and the presence of T2DM should be taken into consideration.

Our study showed a significant correlation between age and COL4-7S in both NAFLD groups. The correlation coefficient, however, was weak in NAFLD without T2DM and there was a poor correlation in NAFLD with T2DM. The correlation between age and COL4-7S was weaker than that between age and the platelet count. COL4-7S reflects the fibrosis process of synthesis and degradation in the liver.<sup>(43,44)</sup> Therefore, COL4-7S may be expected to not be greatly affected by aging. In our study, the platelet count and COL4-7S were only a little affected by aging in practice. However, there are normally no changes in the platelet count or COL4-7S with older age in healthy people.<sup>(44,45)</sup> The reason why COL4-7S was affected by age, although a little, was that subjects in this study were patients with NAFLD and liver fibrosis, and these parameters were affected by progression of liver fibrosis with aging. The present NITs are diagnostic methods for advanced fibrosis  $\geq$  stage 3, but they may be necessary to identify it and screen from stage F2 for intervention and a cure. A model study reported that the cost-effectiveness was better at identifying the early stage of F2 and intervening in patients' lifestyles with vibration-controlled transient elastography than with the FIB-4 index identifying advanced fibrosis and starting medications.<sup>(46)</sup> Identifying fibrosis stage F2 in the future may be important.

This study has several limitations that need to be acknowledged. First, this was a cross-sectional study; therefore, estimating the diagnostic accuracy of COL4-7S was necessary. Second, the efficacy of

COL4-7S has been shown in previous studies, but its efficacy was not validated in our study because of the limitation of a daily blood test as the hepatic fibrosis marker in our daily clinical practice. An international, multicenter, validation study is required to confirm our conclusions. Third, a recent report showed that the amount of domain of collagen in liver fibrosis is related to patients' convalescence<sup>(47)</sup>; however, our study could not consider this because it was a multicenter joint research study, and the specimens were not able to be stained again. The collagen proportionate area of biopsy specimens and its relation to COL4-7S need to be examined in the future. Fourth, the usefulness of diagnosing fibrosis with imaging methods, such as ultrasound elastography and magnetic resonance imaging, instead of liver biopsy has been reported; however, in our study, the availability of these equipment varied between facilities, so we were not able to compare the imaging methods with NITs. Examination comparing these methods with NITs will be necessary for the future. Finally, because this is a cross-sectional study that estimates the relations between NITs and liver fibrosis at the time of liver biopsy, we cannot evaluate an effect of the diabetic medicine for liver fibrosis and the levels of NITs. It will be necessary to examine the influence of the DM medicine on liver fibrosis by a longitudinal study in future.

In conclusion, COL4-7S measurement might be the best NIT to predict advanced fibrosis of NAFLD, especially in NAFLD with T2DM. The diagnostic accuracy of conventional NITs, such as the FIB-4 index, NFS and APRI, is inferior to that of COL4-7S in NAFLD with T2DM.

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## Supporting Information

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep4.1637/supinfo](https://onlinelibrary.wiley.com/doi/10.1002/hep4.1637/supinfo).