



ORIGINAL ARTICLE

A new detection system for serum fragmented cytokeratin 18 as a biomarker reflecting histologic activities of human nonalcoholic steatohepatitis

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Abstract

Caspase-generated fragmented cytokeratin 18 (fCK18) is recognized as a useful noninvasive biomarker in the diagnosis of nonalcoholic fatty liver disease (NAFLD), particularly nonalcoholic steatohepatitis (NASH). However, fCK18 measurement is not applied clinically due to widely variable cut-off values under the current enzyme-linked immunosorbent assay platform. Therefore, we developed a highly sensitive chemiluminescent enzyme immunoassay using newly developed monoclonal antibodies against fCK18 and investigated its relevance in NASH diagnosis. Serum fCK18 levels were measured in the derivation and validation cohort. The correlation between serum fCK18 levels and NAFLD activity score (NAS), fibrosis stage, and liver function was examined. Serum fCK18 levels were significantly correlated with alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase. Serum fCK18 levels were significantly associated with NAS, Brunt's grade/stage, Matteoni's classification, portal inflammation, and fat accumulation in the liver. Notably, hepatocyte ballooning was the only independent variable significantly associated with serum fCK18 in the multivariate linear regression analysis. Serum fCK18 levels were significantly elevated in patients with NAFLD and nonalcoholic fatty liver (NAFL) compared to healthy individuals. They were also significantly elevated in patients with NAFL compared to NASH defined by NAS or Matteoni's classification, with area under the curve values being 0.961 (NAFLD vs. healthy), 0.913 (NAFL vs. healthy), 0.763 (NASH vs. NAFL), and 0.796 (NASH type 3–4 vs. NAFL type 1–2). These results were confirmed by a validation cohort.

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Notably, changes over time in serum fCK18 levels were significantly correlated with changes in ALT, AST, and the fibrosis-4 index in 25 patients who underwent lifestyle modification. Serum fCK18 levels were significantly correlated with liver damage associated with NASH pathology. Serum fCK18 levels are accurate in distinguishing patients with NAFL or NASH from healthy individuals and may be useful to monitor NASH over time.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in both children and adults and is now the fastest growing indicator for liver transplantation in the world—particularly in the United States, Europe, and the United Kingdom.^[1,2] NAFLD is closely associated with obesity and encompasses a wide spectrum of conditions associated with the overaccumulation of fat in the liver, including hepatic steatosis, steatohepatitis (NASH), and cirrhosis. NASH is a serious condition in which approximately 5%–25% of patients will progress to hepatic fibrosis and cirrhosis with associated complications, such as portal hypertension, liver failure, and hepatocellular carcinoma (HCC).^[3] Notably, current reports indicate that cirrhosis is not a prerequisite for HCC in patients with NASH, which is in contrast to patients infected with hepatitis B virus (HBV) or hepatitis C virus,^[4,5] although HCC can develop in patients infected with HBV but without cirrhosis in some cases.^[6]

Liver biopsy is an invasive procedure that can cause potentially significant complications. However, a liver biopsy remains the only reliable method to differentiate hepatic steatosis from NASH, determine the stage and grade of the disease, and monitor patient response to therapeutic interventions.^[4] Liver biopsy is limited to infrequent implementation and is prone to sampling errors^[7,8]; thus, there exists a great unmet need for noninvasive procedures to assist clinicians in the diagnosis of NAFLD. In diagnosing NASH and liver fibrosis, current imaging technologies, including FibroScan and magnetic resonance elastography, enable an accurate assessment of fibrosis but may lack the resolution to detect steatohepatitis.^[9,10] Hepatocyte death by apoptosis is a key event linked to the progression from steatosis to NASH with consequent fibrosis.^[11] During the process of apoptosis, we see activated caspases, mainly caspase 3, cleave a number of cytoplasmic proteins, including cytokeratin 18 (CK18), which is an intermediate filament component of epithelial cells.^[12,13] CK18 is cleaved by caspase-6, yielding a 26-kD N-terminal fragment and a 22-kD C-terminal fragment. The 22-kD C-terminal fragment is then further cleaved by caspase-3 and caspase-7, resulting in a 19-kD fragment.^[12] *In vitro* studies have shown that cells undergoing apoptosis

release soluble CK18 into the extracellular environment and CK18 is further circulated in the blood.^[14] Indeed, caspase-generated CK18 fragments are recognized as an accurate, noninvasive biomarker sufficient to diagnose the progression of NAFLD, particularly the transition from nonalcoholic fatty liver (NAFL) to NASH.^[15–17] Although a commercially available enzyme-linked immunosorbent assay (ELISA) kit specifically designed to measure CK18 fragments in the blood is available and is widely used in the diagnosis of NASH, this assay is not implemented in the clinical setting for several reasons: variability of disease marker cut-off values,^[18,19] no clear delineation between patients with NAFL and healthy individuals,^[20,21] and a limitation in the isolated value of serum CK18 fragments in the diagnosis of NASH due to hepatocyte ballooning not being taken into account.^[22] Therefore, we have developed a highly sensitive chemiluminescent enzyme immunoassay (CLEIA) for the detection of fragmented CK18 (fCK18) using new monoclonal antibodies targeted to CK18 and/or fCK18 and have shown that our new highly sensitive CLEIA system is a simple and reliable detection apparatus^[23] when compared to the existing ELISA. The aim of this study was to use a highly sensitive CLEIA to assess the utility of fCK18 measurements in the determination of NASH, assess disease severity, and monitor disease status in patients with NAFLD who received current lifestyle alteration recommendations and interventions.

MATERIALS AND METHODS

Human samples

The study protocol was approved by the ethics committee of Mie University. This study was performed retrospectively on stored samples. Patients (n = 54) underwent liver biopsy as part of standard clinical procedure as the derivation cohort. The diagnosis of NAFLD was based on liver biopsy features as assessed by two experienced hepatic histopathologists (Y.H. and A.H.).

The retrospective validation cohort serum samples were collected at a different institution (Tokyo University) retrospectively, which was approved by the ethics committee of Tokyo University. Patients (n = 67) underwent

liver biopsy as part of standard clinical procedure. The diagnosis of NAFLD was based on liver biopsy features in which steatosis was present in greater than 5% of hepatocytes. The absence of current excessive alcohol use was defined by an average daily alcohol consumption of <30g/day for men and <20g/day for women. Prevalence of diabetes, hypertension, and hyperlipidemia was assessed based on a review of medical history. We also retrospectively investigated the association between serum fCK18 levels and liver function in the derivation cohort. All patients with NAFLD received recommendations for interventions to alter their lifestyle as outpatients, were followed up every 3 months, and had regular blood collection at each hospital visit. They were educated about dietary restrictions by a dietitian and advised on taking 150 minutes of moderate exercise per week. The study duration was 1197 days (median) between sample collections. Consequently, 29 participants were excluded from this study on the grounds of using medication or loss of follow-up or preserved serum. Serum from healthy individuals was purchased from Precision Medicine group (Maryland, USA) with approval. Serum from healthy individuals was confirmed based on an alanine aminotransferase (ALT) assay being within the normal range. Serum was stored at -80°C .

Liver histology

The histologic diagnosis of NAFLD was performed by the study pathologists according to their expertise and based on the Nonalcoholic Steatohepatitis Clinical Research Network Scoring System commonly used to assess stage of disease severity.^[24] The pathologists were blinded to fCK18 measurements as well as patient clinical and laboratory data. In this scoring system, the degree of steatosis, liver injury, and inflammatory activity was measured using an 8-point scale (steatosis, 0–3; lobular inflammation, 0–3; ballooning degeneration of hepatocytes, 0–2). The NAFLD activity score (NAS) is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores. Cases with $\text{NAS} \leq 2$ were diagnosed as NAFL, while cases with $\text{NAS} 5\text{--}8$ were diagnosed as NASH. Cases with NAS of 3 and 4 were considered borderline NASH.^[24,25] In addition, NAFLD was defined according to Matteoni's classification^[26] and Brunt's grade/stage.^[27] Cases with Matteoni's classification of type 3–4 were considered NASH.

Measurement of fCK18 and other factors in the blood

Blood samples were collected within 24 hours of liver biopsy and measured for ALT, aspartate aminotransferase

(AST), albumin (ALB), gamma-glutamyl transpeptidase ($\gamma\text{-GT}$), total bilirubin (T-bil), sodium (Na), blood urea nitrogen (BUN), and creatinine (Cr). Serum samples within 14 days of liver biopsy were kept at -80°C until fCK18 measurements took place using the HISCL-5000 CLEIA system (Sysmex Corporation, Japan)^[23] according to the manufacturer's instructions. Additionally, serum fCK18 levels were measured at two different time points in those patients with available serum ($n = 25$). The fibrosis index based on four factors (fibrosis-4 [FIB-4]) was calculated.

Statistical analyses

All data are expressed as mean \pm SEM or median and 25th and 75th percentiles for continuous factors. Data were analyzed using Mann–Whitney U test for two groups and Kruskal–Wallis test or chi-square test for three groups. An association between fCK18 and clinical parameters was determined by the Spearman rank-sum test. Multivariate linear regression analyses were performed to evaluate the relationship between fCK18 and pathologic parameters. The independent variables calculated in the multivariate linear regression analysis included ballooning, steatosis, lobular inflammation, and fibrosis. For each continuous variable, the optimal cut-off value that maximized the sum of sensitivity and specificity was selected using receiver operating characteristic (ROC) curve analysis. Statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA, USA) and SPSS Statistics 27.0 (IBM, Armonk, NY, USA). Differences were considered to be significant at $p < 0.05$.

RESULTS

Patient population characteristics

The main clinical and laboratory patient characteristics are shown in Table 1, and liver biopsy histologic characteristics are summarized in Table 2. Patient age (median, 62.5 years), sex (44.4% men), and body mass index (BMI) (median, 26.1 kg/m^2) were not statistically different among the three histologic groups, which are NAFL, borderline NASH, and NASH (Table 1). We found no difference in the prevalence of diabetes, dyslipidemia, or hypertension among the three groups (Table 1). Serum ALT and AST levels were significantly different among the three histologic groups (parameter median in NAFL, borderline NASH, and NASH: AST 24.0, 45.0, and 72.5 U/L, respectively, $p = 0.00257$; ALT 28.0, 41.0, and 67.0 U/L, respectively, $p = 0.0087$) (Table 1). We observed an increasing FIB-4 index scores trend from NAFL to borderline

TABLE 1 Demographic and clinical characteristics of subjects

Parameter	All (n = 54)	NAFL (n = 5)	Borderline NASH (n = 19)	NASH (n = 30)	p value ^a
Age (years)	62.5 (55.3, 70.3)	68.0 (50.0, 75.0)	64.0 (56.0, 72.0)	61.5 (53.0, 70.0)	0.7639
Sex (M/F)	24/30	1/4	7/12	16/14	0.2705
BMI (kg/m ²)	26.1 (24.2, 29.4)	26.1 (22.0, 26.9)	25.1 (23.2, 29.5)	26.1 (24.9, 31.0)	0.3018
AST (U/L)	52.5 (33.0, 79.0)	28.0 (22.0, 72.5)	41.0 (23.0, 66.0)	67.0 (48.0, 101.3)	0.0087
ALT (U/L)	54.5 (32.0, 91.0)	24.0(20.5, 89.5)	45.0 (18.0, 69.0)	72.5 (47.8, 118.0)	0.0257
ALB (g/dl)	4.35 (4.10, 4.60)	4.40 (4.20, 4.40)	4.30 (4.10, 4.60)	4.35 (4.00, 4.60)	0.9276
T-bil (mg/dl)	0.70 (0.58, 1.00)	0.60 (0.50, 0.85)	0.80 (0.60, 1.30)	0.70 (0.50, 1.00)	0.3009
γ-GT (U/L)	55.0 (27.0, 89.5)	160.0 (38.0, 206.0)	39.0 (27.0, 87.0)	55.5 (38.0, 86.8)	0.2783
FIB-4 index	2.47 (1.52, 3.58)	1.53 (1.24, 2.19)	1.92 (1.50, 3.23)	2.75 (1.65, 5.14)	0.1002
Na (mEq/L)	141.0 (140.0, 142.0)	142.0 (140.0, 143.0)	141.0 (140.0, 142.0)	141.0 (140.0, 142.8)	0.8268
BUN (mg/dl)	13.4 (10.9, 16.1)	16.2 (12.5, 17.8)	13.8 (10.0, 15.7)	12.8 (10.9, 16.0)	0.4130
Cr (mg/dl)	0.65 (0.58, 0.80)	0.72 (0.54, 0.96)	0.63 (0.56, 0.81)	0.65 (0.59, 0.81)	0.9369
Diabetes (no/yes)	33/20	3/2	13/6	17/12	0.7861
Dyslipidemia (no/yes)	36/17	3/2	11/8	22/7	0.3945
Hypertension (no/yes)	25/28	2/3	9/10	14/15	0.9429

Note: Statistics include number (%) or median (25th and 75th percentiles). Kruskal-Wallis test for patient number for diabetes, dyslipidemia, and hypertension. Chi-square test for sex, diabetes, dyslipidemia, and hypertension.

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; F, female; FIB-4, fibrosis-4; γ-GT, gamma-glutamyl transpeptidase; M, male; Na, sodium; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; T-bil, total bilirubin.

^a*p* < 0.05 is statistically significant.

TABLE 2 Histologic characteristics of the patient population

Factor	Number (%)	Factor	Number (%)
Steatosis		NAS	
0 (<5%)	3 (5.6)	NAFL	
1 (5–33%)	14 (25.9)	0	1 (1.9)
2 (>33–66%)	26 (48.1)	1	2 (3.7)
3 (>66%)	11 (20.4)	2	2 (3.7)
Lobular Inflammation	Borderline NASH		
0–1 (<2 foci/20× field)	28 (51.8)	3	9 (16.6)
2 (2–4 foci/20× field)	23 (42.6)	4	10 (18.5)
3 (>4 foci/20× field)	3 (5.6)	NASH	
Ballooning	5	15 (27.8)	
0 (None)	11 (20.4)	6	12 (22.2)
1 (Few)	25 (46.3)	7	1 (1.9)
2 (Many)	18 (33.3)	8	2 (3.7)
Fibrosis			
1	20 (37.0)		
2	18 (33.3)		
3	15 (27.8)		
4	1 (1.9)		

Abbreviations: NAFL, nonalcoholic fatty liver; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis.

NASH and NASH (median in NAFL, borderline NASH, and NASH: 1.53, 1.92, and 2.75, respectively), although these data were not significant (Table 1). In

contrast, serum ALB, T-bil, γ-GT, Na, BUN, and Cr levels were not significantly changed among the three histologic groups (Table 1).

Serum fCK18 levels are significantly correlated with liver enzyme levels

We examined the correlation between fCK18 measurements and clinical parameters. Serum fCK18 levels were significantly correlated with ALT ($r = 0.722$, $p < 0.0001$), AST ($r = 0.737$, $p < 0.0001$), and γ-GT ($r = 0.518$, $p < 0.0001$) (Figure 1A–C). Serum fCK18 levels were weakly associated with FIB-4 index scores ($r = 0.250$, $p = 0.068$). In contrast, we found no significant correlation between serum fCK18 levels and the following parameters: age ($r = -0.233$, $p = 0.089$), BMI ($r = 0.234$, $p = 0.091$), ALB ($r = 0.152$, $p = 0.274$), T-bil ($r = 0.240$, $p = 0.081$), Na ($r = -0.097$, $p = 0.504$), BUN ($r = -0.262$, $p = 0.056$), and Cr ($r = -0.009$, $p = 0.950$). These results indicate that serum fCK18 levels reflect the degree of liver damage.

Serum fCK18 levels are significantly increased in patients with NASH

Next, we investigated whether patient serum fCK18 levels are associated with liver pathology in human NAFLD, including NAFL, borderline NASH, and NASH. Serum fCK18 levels gradually increased following the

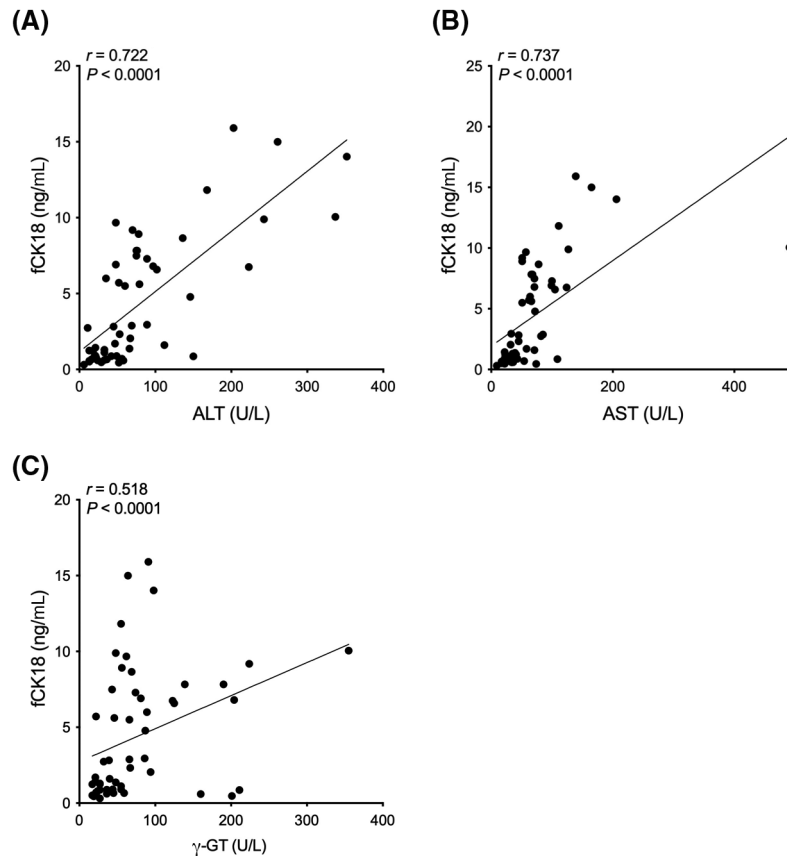


FIGURE 1 Serum fCK18 levels are correlated with liver function in patients with NAFLD. Correlation between serum fCK18 levels and (A) ALT, (B) AST, and (C) γ -GT. ALT, alanine aminotransferase; AST, aspartate aminotransferase; fCK18, fragmented cyokeratin-18; γ -GT, gamma-glutamyl transpeptidase; NAFLD, nonalcoholic fatty liver disease

progression of NAFLD, from NAFL (NAS, 0–2: median, 0.864 ng/ml) to borderline NASH (NAS, 3–4: median, 1.242 ng/ml) and NASH (NAS, 5–8: median, 6.290 ng/ml), with a particularly significant elevation observed in patients with NASH (NAFL or borderline NASH vs. NASH, $p < 0.01$) (Figure 2A). When we analyzed each NAS factor independently, we found significantly elevated serum fCK18 levels in patients with NAFLD with moderate (stage 2: median, 5.857 ng/ml) and severe (stage 3: median, 2.876 ng/ml) steatosis than in those with none (stage 0: median, 0.596 ng/ml) and mild (stage 1: median, 1.272 ng/ml) steatosis (stage 0 vs. 2 or 3, $p < 0.05$) (Figure 2B). Serum fCK18 levels were dramatically elevated in patients with NAFLD with hepatocyte ballooning scores of “few” (stage 1: median, 2.950 ng/ml) or “many” (stage 2: median, 6.663 ng/ml) when compared to samples displaying an absence of hepatocyte ballooning (stage 0: median, 0.869 ng/ml) (stage 0 vs. 1, $p < 0.01$; 0 vs. 2, $p < 0.001$) (Figure 2C). Serum fCK18 levels were higher in patients with NAFLD with mild (stage 2: median, 5.709 ng/ml) or moderate (stage 3: median, 10.05 ng/ml) lobular inflammation than in those with none (stage 0–1: median, 1.182 ng/ml) (Figure 2D). These results indicate

that serum fCK18 levels are useful in the diagnosis of human NASH, including hepatocyte ballooning.

Serum fCK18 levels are significantly associated with various NAFLD diagnostic scoring systems

The NAS score is composed of three factors—steatosis, lobular inflammation, and hepatocyte ballooning—with liver fibrosis being notably absent. Therefore, we explored the association between serum fCK18 levels and other NASH scoring platforms, such as Brunt's grade/stage and Matteoni's classification, which include liver fibrosis as part of their scoring system. Serum fCK18 levels were significantly associated with Brunt's grade from none (grade 0: median, 0.869 ng/ml) to mild (grade 1: median, 2.818 ng/ml), moderate (grade 2: median, 5.857 ng/ml), and severe (grade 3: median, 12.98 ng/ml) (grade 0 vs. 1 and 1 vs. 3, $p < 0.05$; 0 vs. 2 or 3, $p < 0.01$) (Figure 3A). In looking at liver fibrosis as a criterion of the Brunt scoring system, serum fCK18 levels were significantly higher in patients with NAFLD, with fibrosis observed at the central vein and

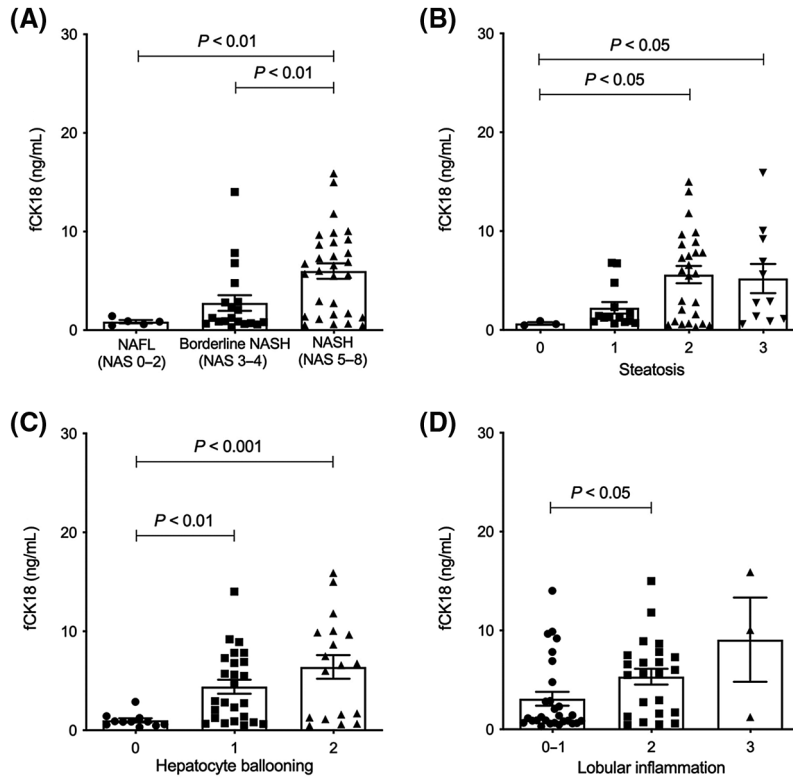


FIGURE 2 Serum fCK18 levels are associated with NASH. Serum fCK18 levels in (A) NASH, (B) steatosis, (C) hepatocyte ballooning, and (D) lobular inflammation. Values are mean ± SEM. fCK18, fragmented cytoke­ ratin-18; NAFL, nonalcoholic fatty liver; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis

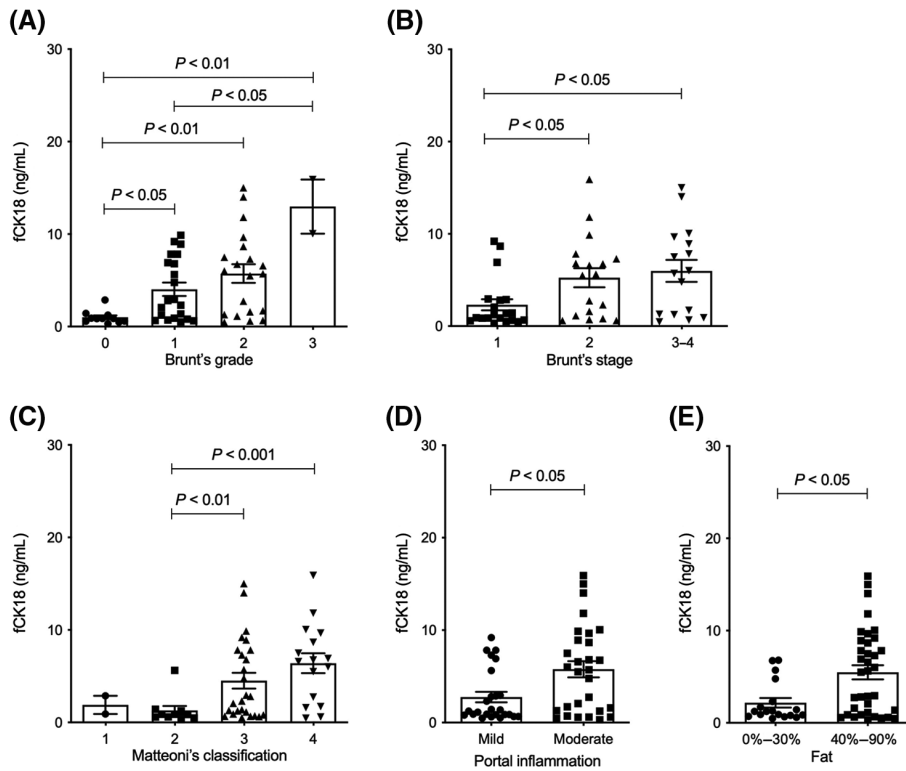


FIGURE 3 Serum fCK18 levels are associated with other NASH scoring systems. Serum fCK18 levels in (A) Brunt's grade, (B) Brunt's stage, (C) Matteoni's classification, (D) portal inflammation, and (E) fat. Values are mean ± SEM. fCK18, fragmented cytoke­ ratin-18; NASH, nonalcoholic steatohepatitis

portal area (stage 2: median, 5.558 ng/ml) and bridging fibrosis–cirrhosis (stage 3–4: median, 5.857 ng/ml) than in those patients with fibrosis present at the central vein (stage 1: median, 1.076 ng/ml) (stage 1 vs. 2 or 3–4, $p < 0.05$) (Figure 3B), suggesting that serum fCK18 levels are increased in patients with liver fibrosis. Using Matteoni's classification method, serum fCK18 levels did not differ between fatty liver (type 1: median, 1.893 ng/ml) and fatty liver with inflammation (type 2: median, 0.866 ng/ml). However, fCK18 levels significantly increased in patients with type 2 Matteoni's classification coupled with hepatocyte ballooning (type 3: median, 2.567 ng/ml) and liver fibrosis plus infiltrated inflammatory cells with type 3 Matteoni's classification (type 4: median, 6.663 ng/ml) (type 2 vs. 3, $p < 0.01$; 2 vs. 4, $p < 0.001$) (Figure 3C), suggesting that serum fCK18 levels reflect overall degree of hepatocyte ballooning with the sample. In analyzing the specific Brunt criteria of portal inflammation and fat composition, serum fCK18 levels were found to be higher in patients with NAFLD with moderate portal inflammation (median, 5.709 ng/ml) than in those with mild portal inflammation (median, 1.242 ng/ml) ($p < 0.05$) (Figure 3D) and in patients with 0%–30% fat (median, 1.243 ng/ml) compared to those with 40%–90% fat (median, 5.558 ng/ml) ($p < 0.05$) (Figure 3F). These results indicate that serum fCK18 levels are associated with various NAFLD scoring systems.

Ballooning shows a significant relationship with serum fCK18 among pathologic parameters

To determine which pathologic parameter has the strongest relationship with serum fCK18, we performed multivariate linear regression analyses using ballooning, steatosis, lobular inflammation, and fibrosis as independent variables. Ballooning was the only independent variable to be significantly associated with serum fCK18 levels (standard $\beta = 0.452$, $p < 0.001$), while other variables, such as steatosis, lobular inflammation, and fibrosis, were not significantly correlated with serum fCK18 levels (Table 3). This result reveals

TABLE 3 Multivariate linear regression analysis detailing the relationship between fCK18 and pathologic parameters

Independent variables	Standard β	p value ^a
Ballooning	0.452	<0.001
Steatosis	0.222	0.086
Lobular inflammation	0.222	0.131
Fibrosis	0.167	0.254

Note: For multivariate analysis, decision coefficient (R^2) = 0.204, $p < 0.001$.

Abbreviation: fCK18, fragmented cytokeratin 18.

^a $p < 0.05$ is statistically significant.

that serum fCK18 levels strongly reflect hepatocyte ballooning.

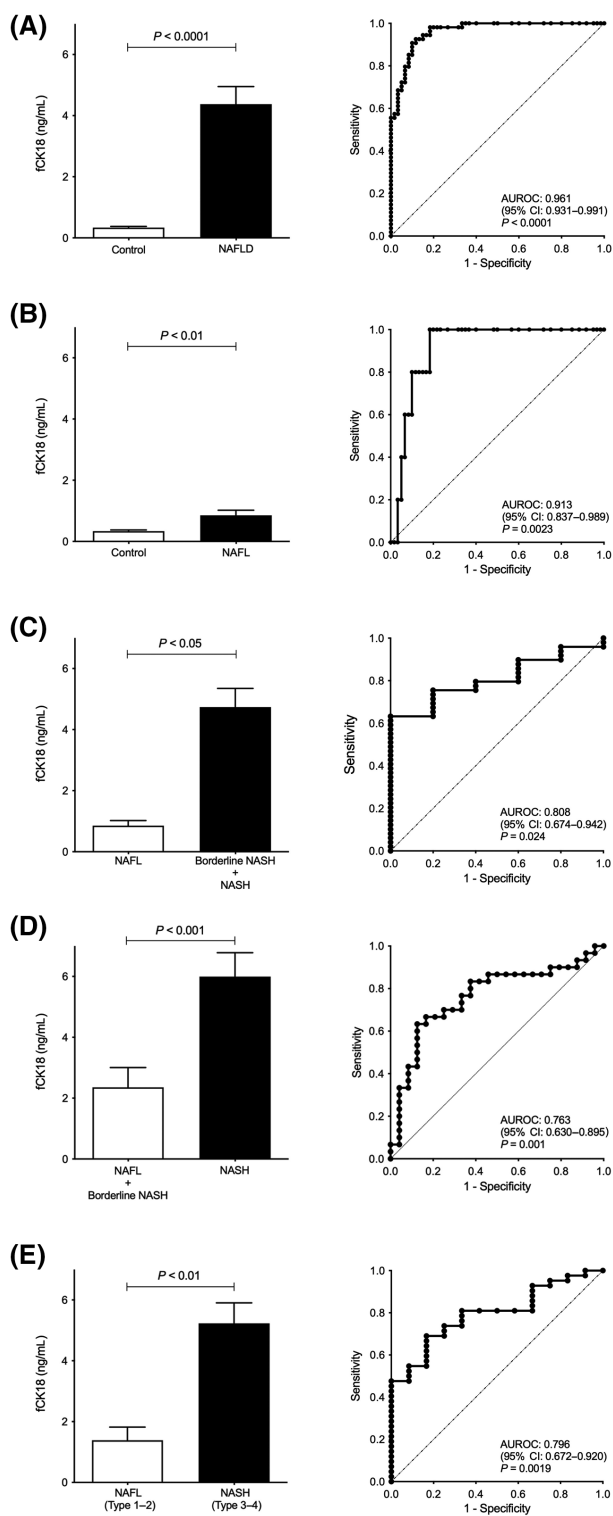
Serum fCK18 levels can be used as a predictor of NAFL/NASH

Serum fCK18 levels were significantly increased in all patients with NAFLD when compared to healthy individuals (NAFLD vs. healthy: median, 2.528 vs. 0.250 ng/ml; $p < 0.0001$) (Figure 4A). ROC analyses yielded area under the curve (AUC) values of 0.961 for serum fCK18 levels (Figure 4A; Table 4). In separating patients with NAFL from the healthy population, we found serum fCK18 levels significantly increased in patients with NAFL compared to healthy individuals (NAFL vs. healthy: 0.864 vs. 0.250 ng/ml, $p = 0.0023$) (Figure 4B). ROC analyses yielded AUC values of 0.913 for serum fCK18 levels (Figure 4B; Table 4). In our effort to determine fCK18 cut-off values, we found 0.57 ng/ml and 0.46 ng/ml as threshold values able to distinguish healthy individuals from all patients with NAFLD and patients with NAFL, respectively (Table 4). Finally, we sought to determine the cut-off value able to distinguish patients with borderline NASH plus NASH from patients with NAFL. Notably, serum fCK18 levels were significantly elevated in patients with borderline NASH plus NASH when compared with patients with NAFL (NAFL vs. borderline plus NASH: 0.864 vs. 2.876 ng/ml, $p = 0.022$) (Figure 4C). ROC analyses yielded AUC values of 0.808 for serum fCK18 levels (Figure 4C; Table 4), and the cut-off value was 1.0 ng/ml to distinguish borderline plus NASH from patients with NAFL (Table 4). When we performed a side by side comparison of NAFL and NASH samples, we found serum fCK18 levels were significantly elevated in patients with NASH with NAS 5–8 (NAFL plus borderline NASH vs. NASH: 0.910 vs. 6.290 ng/ml, $p = 0.0008$) (Figure 4D). In our effort to distinguish patients with NASH from patients with NAFL, we determined a cut-off value of 5.2 ng/ml, with an AUC value of 0.763 (Table 4).

The Matteoni's classification is another scoring system able to distinguish NASH from NAFL; therefore, we compared our patient samples of NAFL (type 1–2) and NASH (type 3–4) using this scoring method. We determined that serum fCK18 levels were significantly elevated in patients with NASH (type 3–4) (NAFL type 1–2 vs. NASH type 3–4: 0.873 vs. 5.138 ng/ml, $p = 0.0014$) (Figure 4E). The observed cut-off value to distinguish patients with NASH (type 3–4) from patients who were type 1–2 was 1.6 ng/ml, with an AUC value of 0.796 (Table 4). These results indicate that serum fCK18 measured by our new assay can be used to distinguish patients with NAFL and patients with NASH from healthy individuals as well as patients with NASH from patients with NAFL.

Validation cohort: Serum fCK18 levels are useful for NASH diagnosis, particularly hepatocyte ballooning

We further investigated the association between serum fCK18 levels and liver histology, using a validation cohort of 67 patients. The main clinical and laboratory patient characteristics are shown in Table 5, and liver biopsy



histologic characteristics are summarized in Table 6. Serum ALT, AST, and γ -GT levels differed significantly among the three histologic groups. Serum fCK18 levels gradually increased in line with the progression of NAFLD (NAFL, borderline NASH, to NASH), with a particularly significant elevation observed in patients with NASH (NAFL vs. borderline NASH, $p < 0.05$; NAFL or borderline NASH vs. NASH, $p < 0.0001$ or $p < 0.01$, respectively) (Figure 5A). When analyzing each NAS factor independently, we observed elevated serum fCK18 levels in patients with NAFLD with mild, moderate, and severe steatosis when compared to patients with no observed steatosis (Figure 5B). Serum fCK18 levels were dramatically elevated in patients with NAFLD presenting with hepatocyte ballooning scores of few or many when compared to patients with no observable hepatocyte ballooning (stage 0 vs. 1 or 2, $p < 0.01$ or $p < 0.001$, respectively) (Figure 5C). Serum fCK18 levels were significantly higher in patients with NAFLD with mild lobular inflammation when compared to those with none (stage 0–1 vs. 2, $p < 0.001$) (Figure 5D). When taking liver fibrosis into consideration as a criterion of the Brunt scoring system, we found serum fCK18 levels to be significantly higher in patients with NAFLD with fibrosis, whether at the central vein, the central vein and portal area, or bridging fibrosis–cirrhosis, when compared to patient samples lacking observable fibrosis (stage 0 vs. 1 or 3–4, $p < 0.05$) (Figure 5E). Within Matteoni's classification system, serum fCK18 levels were found to be increased in fatty liver with inflammation, hepatocyte ballooning with type 2 Matteoni's classification, and liver fibrosis plus infiltrated inflammatory cells with type 3 Matteoni's classification when compared to samples presenting with fatty liver alone (type 2 vs. 4, $p < 0.01$) (Figure 5F). Furthermore, ROC analyses calculations determined that serum fCK18 levels were significantly elevated in patients with borderline NASH plus NASH when compared with patients with NAFL, with AUC values of 0.818 ($p = 0.0023$) (Figure 5G). When we compared between NAFL and NASH, serum fCK18 levels were significantly elevated in patients with NASH (NAS 5–8), with an AUC value of 0.769 ($p = 0.0004$)

FIGURE 4 Serum fCK18 levels are increased in patients with NAFLD. Serum fCK18 levels or ROC curve of serum fCK18 levels between (A) patients with NAFLD and control (healthy individuals) or (B) patients with NAFL and control. (C) Serum fCK18 levels or ROC curve of serum fCK18 levels between patients with NAFL and patients with borderline NASH plus NASH. (D) Serum fCK18 levels or ROC curve of serum fCK18 levels between patients with NAFL plus borderline NASH and patients with NASH. (E) Serum fCK18 levels or ROC curve of serum fCK18 levels between patients with NAFL (type 1–2) and patients with NASH (type 3–4). Values are mean \pm SEM. AUROC, area under the receiver operating characteristic curve; CI, confidence interval; fCK18, fragmented cytochrome-18; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ROC, receiver operating characteristic; Type 1–2, Matteoni's classification type 1–2; Type 3–4, Matteoni's classification type 3–4

TABLE 4 Summary of ROC analysis

Comparison	AUC	95% CI	Sensitivity	Specificity	Cutoff	p value ^a
Control						
vs. NAFLD	0.961	0.931–0.991	92.6%	88.3%	0.57 ng/mL	<0.0001
vs. NAFL	0.913	0.837–0.989	100%	81.7%	0.46 ng/mL	<0.0001
NAFL						
vs. Borderline NASH+NASH	0.808	0.674–0.992	75.5%	80.0%	1.0 ng/mL	0.024
NASH						
vs. NAFL + borderline NASH	0.763	0.630–0.895	63.3%	87.5%	5.2 ng/mL	0.001
NASH (type 3–4)						
vs. NAFL (type 1–2)	0.796	0.672–0.920	69.1%	83.3%	1.6 ng/mL	0.0019

Note: Type 1–2, Matteoni's classification type 1–2; Type 3–4, Matteoni's classification type 3–4.

Abbreviations: AUC, area under the curve; CI, confidence interval; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ROC, receiver operating characteristic.

^ap < 0.05 is statistically significant.

TABLE 5 Demographic and clinical characteristics of subjects in a validation cohort

Parameter	All (n = 67)	NAFL (n = 9)	Borderline NASH (n = 36)	NASH (n = 22)	p value ^a
Age (years)	53.8 (43.3, 65.0)	60.8 (31.5, 70.9)	54.2 (46.0, 63.2)	51.8 (40.5, 66.9)	0.9745
Sex (M/F)	36/31	6/3	18/18	12/10	0.6659
AST (U/L)	47.0 (30.0, 72.0)	25.0 (22.0, 32.5)	44.5 (29.3, 59.0)	64.5 (48.0, 109.3)	<0.0001
ALT (U/L)	69.0 (43.0, 103.0)	43.0 (22.0, 65.0)	59.5 (39.5, 82.5)	104.0 (78.0, 158.3)	<0.0001
ALB (g/dl)	4.00 (3.70, 4.20)	4.10 (3.45, 4.45)	3.85 (3.60, 4.20)	4.00 (3.70, 4.13)	0.8818
T-bil (mg/dl)	0.80 (0.70, 1.10)	0.80 (0.70, 0.90)	0.80 (0.60, 1.18)	0.80 (0.70, 1.10)	0.9778
γ-GT (U/L)	82.0 (43.0, 131.0)	48.0 (31.5, 97.5)	63.5 (44.0, 100.0)	116.5 (78.8, 191.0)	0.0095 ^a
FIB-4 index	1.15 (0.82, 1.49)	0.85 (0.35, 1.51)	1.02 (0.83, 1.34)	1.37 (0.80, 2.00)	0.0772
Na (mEq/L)	142.0 (140.0, 143.0)	142.0 (140.0, 143.5)	141.5 (140.0, 142.0)	141.5 (140.0, 143.0)	0.6308
BUN (mg/dl)	12.6 (10.8, 15.1)	12.9 (11.3, 15.6)	12.0 (10.4, 14.4)	13.1 (11.2, 16.0)	0.3424
Cr (mg/dl)	0.69 (0.58, 0.85)	0.82 (0.73, 0.99)	0.69 (0.58, 0.83)	0.65 (0.58, 0.84)	0.0657
Diabetes (no/yes)	38/29	5/4	17/19	8/14	0.5656
Dyslipidemia (no/yes)	43/24	3/6	13/23	8/14	0.9859
Hypertension (no/yes)	41/26	2/7	12/24	12/10	0.1502

Note: Statistics include number (%) or median (25th and 75th percentiles). Kruskal-Wallis test for patient number for diabetes, dyslipidemia, and hypertension. Chi-square test for sex, diabetes, dyslipidemia, and hypertension.

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; F, female; FIB-4, fibrosis-4; γ-GT, gamma-glutamyl transpeptidase; M, male; Na, sodium; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; T-bil, total bilirubin.

^ap < 0.05 is statistically significant.

(Figure 5G), and were elevated in NASH (type 3–4), with an AUC value of 0.787 ($p = 0.0008$) (Figure 5G). These validation cohort results indicate that serum fCK18 levels are useful in the diagnosis of human NASH, including hepatocyte ballooning, and in distinguishing patients with NASH from patients with NAFL.

Serum fCK18 levels are significantly correlated with liver enzyme levels

Finally, we investigated the association between serum fCK18 levels, liver enzyme levels, and liver

function using 25 patients with NAFLD who received a recommendation of lifestyle modification. Of the 25 patients with NAFLD, eight (32%) saw an increase in serum fCK18 levels, 16 (64%) saw a decrease, and one (4%) saw no change. We calculated ratios based on the change (delta) in enzyme levels and liver function. Delta ALT and delta AST showed a strong positive correlation with each other and delta FIB-4 index values as well as a weak positive correlation with delta ALB and delta T-bil (Figure 6A). In contrast, delta fCK18 had a mild positive correlation with delta AST, delta ALT, and delta FIB-4 index values, but we observed no correlation between delta ALB and delta

TABLE 6 Histologic characteristics of the patient population in a validation cohort

Factor	Number (%)	Factor	Number (%)
Steatosis		NAS	
0 (<5%)	1 (1.5)	NAFL	
1 (5–33%)	30 (44.8)	0	0 (0.0)
2 (>33–66%)	27 (40.3)	1	2 (3.0)
3 (>66%)	9 (13.4)	2	7 (10.4)
Lobular Inflammation	Borderline NASH		
0–1 (<2 foci/20× field)	49 (73.1)	3	17 (25.4)
2 (2–4 foci/20× field)	17 (25.4)	4	19 (28.3)
3 (>4 foci/20× field)	1 (1.5)	NASH	
Ballooning	5	16 (23.9)	
0 (None)	17 (25.4)	6	5 (7.5)
1 (Few)	35 (52.2)	7	1 (1.5)
2 (Many)	15 (22.4)	8	0 (0.0)
Fibrosis			
0	10 (14.9)		
1	23 (34.3)		
2	9 (13.4)		
3	22 (32.9)		
4	3 (4.5)		

Abbreviations: NAFL, nonalcoholic fatty liver; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis.

T-bil, suggesting that fCK18 reflects liver injury in conjunction with liver enzyme assays. Delta fCK18 levels were significantly correlated with delta ALT ($r = 0.648$, $p = 0.0005$) (Figure 6B) and delta AST ($r = 0.585$, $p = 0.002$) (Figure 6C) levels as well as delta FIB-4 index scores ($r = 0.424$, $p = 0.035$) (Figure 6D). These results suggest that serum fCK18 measured by the highly sensitive CLIEA can be used to monitor overall liver injury status.

DISCUSSION

Our study demonstrates that serum fCK18 levels are significantly correlated with liver enzyme measurements and increase in patients with NASH as defined by NAS. We also showed that serum fCK18 levels are significantly associated with additional NASH scoring systems, such as Brunt's grade/stage and Matteoni's classification. Liver damage, including hepatocyte ballooning, based on the caspase-utilizing mechanism of fCK18 production, can and should be monitored by fCK18 detection systems. Indeed, many studies have demonstrated that fCK18 levels in the blood are associated with liver pathology and are a useful noninvasive biomarker in the diagnosis of NAFLD.^[12,15–17]

A few years after the fCK18 ELISA was manufactured, a number of studies concluded that the fCK18 ELISA was not suitable for use in the clinical setting due to imprecise cut-off values and an overall inaccuracy in detecting hepatocyte ballooning.^[18–22] Due to the aforementioned drawbacks of the current commercially available ELISA system, we felt it was imperative to develop a unique detection system based on our highly sensitive CLEIA. Our CLEIA platform uses new monoclonal antibodies raised against CK18 and/or fCK18 that display a greater level of specificity^[23] when compared to the commercially available anti-CK18 fragment antibody, which has been given the clone name M30. Using our highly sensitive CLIEA, serum fCK18 levels were significantly increased in the hepatocyte ballooning categories of few (stage 1) or many (stage 2) when compared to none (stage 0) and were significantly elevated in Matteoni's classification stages 3 and 4 with hepatocyte ballooning compared to stages 1 and 2 without hepatocyte ballooning. In addition, hepatocyte ballooning was the only independent variable significantly associated with serum fCK18 found in our multivariate linear regression analysis. These results strongly indicate that the degree of hepatocyte ballooning can be measured using our highly sensitive CLEIA, even at early stage NASH; this is far superior to the capabilities of the currently available M30 ELISA kit.^[22]

Current noninvasive imaging platforms, such as FibroScan and magnetic resonance imaging-based systems, have a strong advantage in the noninvasive diagnosis of liver fibrosis (including grade) but are unable to diagnose hepatocellular damage and are interfered with by ascites and subcutaneous fatty tissue. Therefore, the determination of hepatocyte damage and ballooning is critical in the diagnosis of NASH, including early stage NASH.^[18,28] In this study, serum fCK18 levels were found to be significantly higher in patients with NAFLD with Brunt's stage fibrosis of 2–4, resulting in an accurate diagnosis of fibrosis; this reflected the overall condition of the liver, although further studies are required to validate this result. Based on these results, it would appear that the measurement of serum fCK18 levels by a highly sensitive CLEIA is useful in the diagnosis of liver damage, including hepatocyte ballooning, steatosis, fibrosis, and inflammation.

Hepatocellular damage is the key event contributing to the progression of NAFLD; thus, noninvasive biomarkers are required to monitor the entirety of liver/hepatocyte damage beginning at the early stage of NASH. Using a highly sensitive CLEIA, it was determined that serum fCK18 levels were significantly correlated with liver enzyme values, which corroborates previous reports.^[15,22] These results suggest that the measurement of serum fCK18 levels will have a significant impact on routine care monitoring

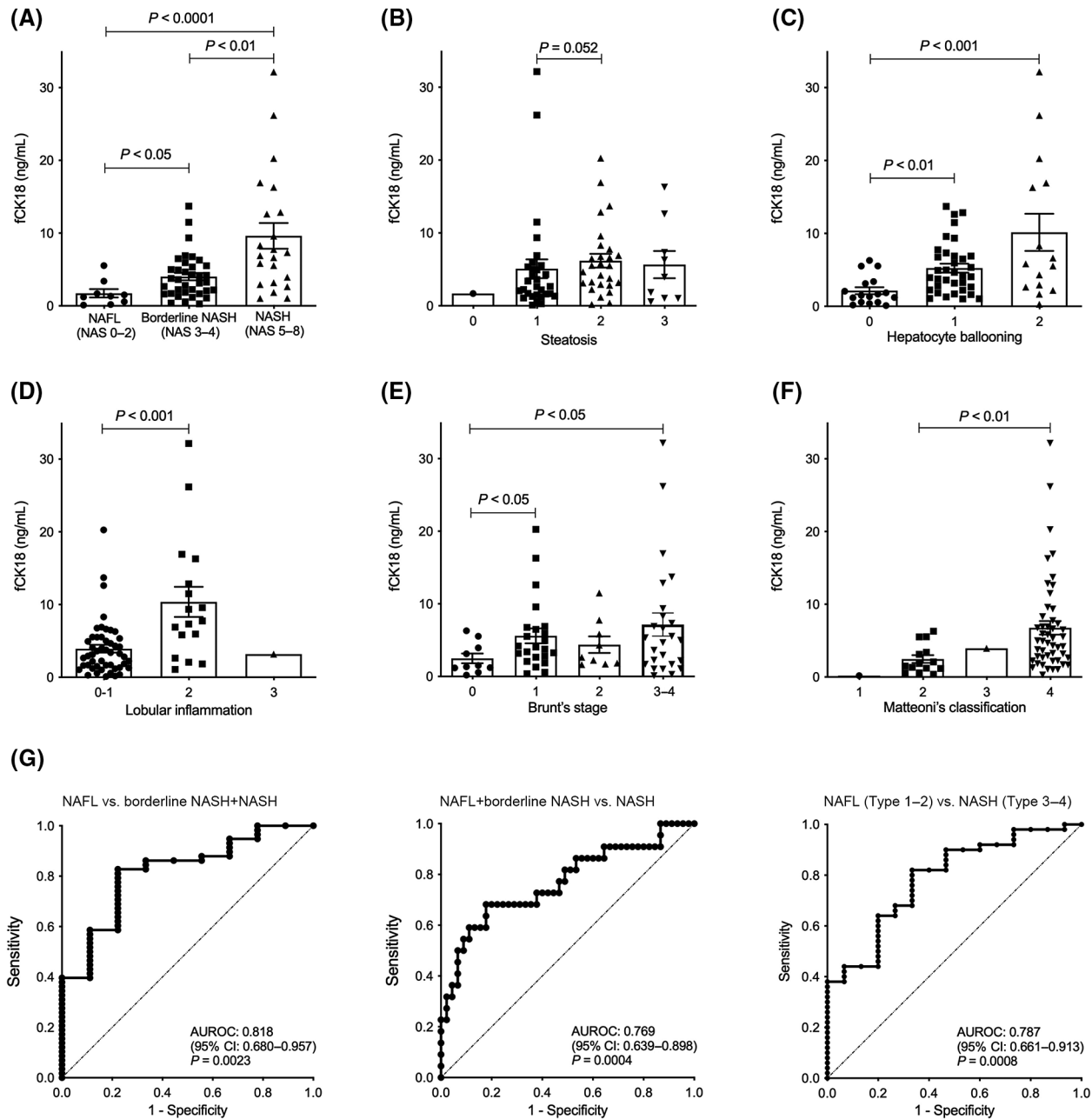


FIGURE 5 Serum fCK18 levels are associated with NAS and increased in patients with NASH in a validation cohort. Serum fCK18 levels in (A) NAS, (B) steatosis, (C) hepatocyte ballooning, (D) lobular inflammation, (E) Brunt's stage, and (F) Matteoni's classification. (G) ROC curve of serum fCK18 levels between patients with NAFL versus patients with borderline NASH plus NASH, patients with NAFL plus borderline NASH versus patients with NASH, and patients with NAFL (type 1–2) versus patients with NASH (type 3–4). Values are mean \pm SEM. AUROC, area under the receiver operating characteristic curve; CI, confidence interval; fCK18, fragmented cytoke-
 ratin-18; NAFL, nonalcoholic fatty liver; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; ROC, receiver operating characteristic; Type 1–2, Matteoni's classification type 1–2; Type 3–4, Matteoni's classification type 3–4

of hepatocyte damage, both short and long term, and may take the place of liver biopsy. In NASH therapeutic clinical trials, liver biopsy, and general blood tests, such as ALT, are the gold standard used to assess liver damage, including steatosis, fibrosis, and inflammation, which are the criteria for assessing drug efficacy. However, more discreet hepatocyte damage and/or hepatocyte recovery will occur

before gross changes to liver pathology. Therefore, the monitoring of hepatocyte damage using serum fCK18 levels is advantageous in determining the efficacy of NASH test drugs instead of the current systems of diagnosis.

We found gradually increased cut-off values of serum fCK18 levels as a way to distinguish patients with NAFL, borderline NASH, and NASH from healthy individuals.

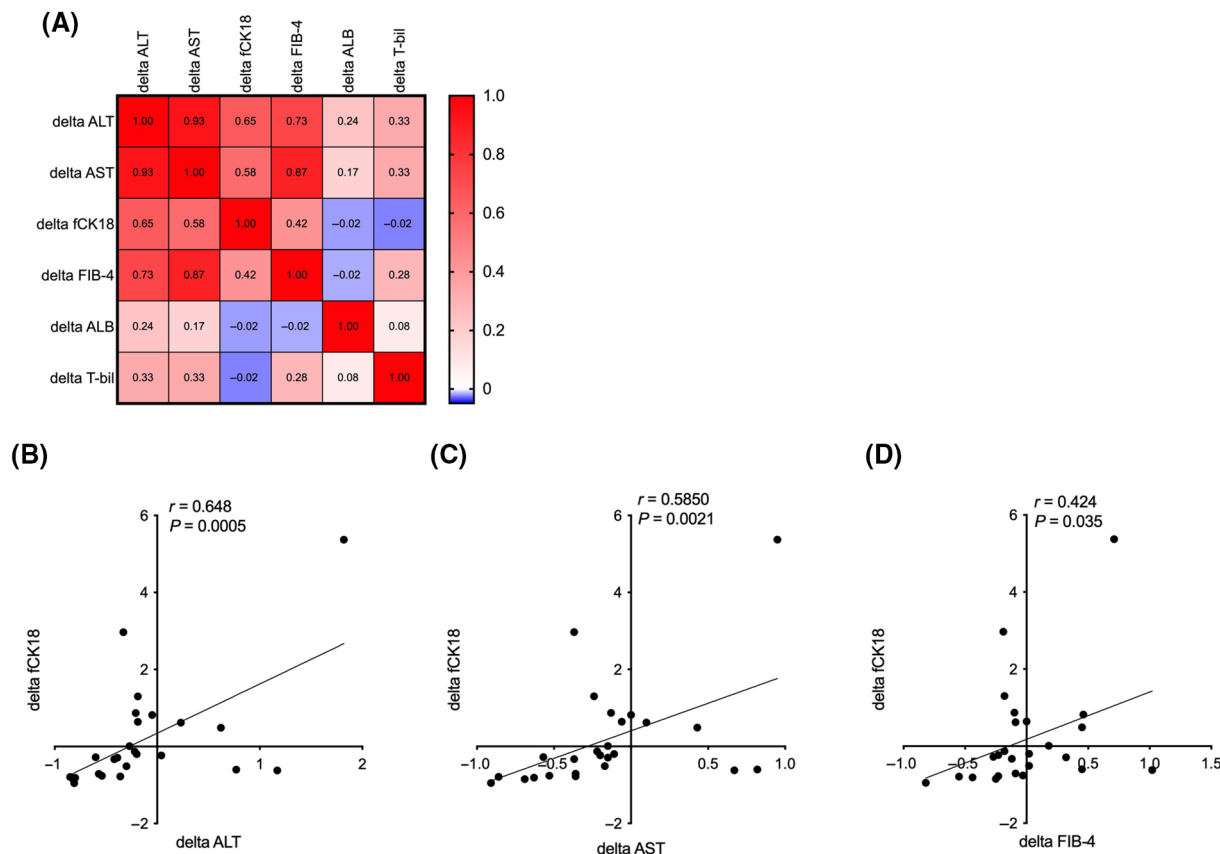


FIGURE 6 Serum fCK18 levels are correlated with liver enzymes. (A) correlation of difference of change ratio (delta) factors. The number indicates spearman r . Delta of serum fCK18 levels with (B) delta ALT, (C) delta AST, (D) delta FIB-4 index. ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; fCK18, fragmented cytoke-
 ratin-18; FIB-4, fibrosis-4; T-bil, total bilirubin

Serum fCK18 levels were significantly increased in patients with NASH with Matteoni's classification stages 3 and 4, which has been found to be associated with a high rate of mortality,^[26] and with Brunt's stages 3 and 4, which are associated with liver fibrosis. In addition, serum fCK18 levels can be used to distinguish patients with borderline plus NASH (NAS 3–8) from patients with NAFL (NAS 0–2). Notably, our results for AUC, sensitivity, and specificity of the cut-off value determined for separating patients with NASH from healthy individuals were higher when compared to the current M30 ELISA figures.^[22] These results indicate that serum fCK18 levels analyzed using our new CLEIA system may be a strong noninvasive tool to diagnose liver fibrosis and to predict overall survival of patients with NAFLD, although we freely admit more analysis is required.

This study is limited insofar as it is retrospective and all samples were collected from only two centers. We need to validate these results, including cut-off values, using a multicenter study approach and a large number of patients with NAFLD, particularly patients with NAFL.

In conclusion, we demonstrated that serum fCK18 levels measured by a highly sensitive CLEIA were significantly correlated with ALT, AST, and γ -GT levels and were significantly associated with the NASH scoring

systems NAS, Brunt's grade/stage, and Matteoni's classification. This newly established assay provides the potential to establish the wider clinical use of serum fCK18 levels in the diagnosis of NAFL and NASH.

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CONFLICT OF INTEREST

Minori Yamada, Koji Okuno, Koji Sakaguchi, and Tetsuji Yamaguchi are employees of Sysmex Co., Ltd. The other authors have nothing to report.

AUTHOR CONTRIBUTIONS

Akiko Eguchi, Minori Yamada, Yoshifumi Hirokawa, Akinobu Hayashi, Koji Okuno, Koji Sakaguchi, and Tetsuji Yamaguchi performed experiments and generated, analyzed, and interpreted data. Akiko Eguchi, Motoshi Iwasa, Minori Yamada, Koji Okuno, Tetsuji Yamaguchi, Yoshiyuki Takei, and Hayato Nakagawa drafted and reviewed the manuscript. Akiko Eguchi, Motoshi Iwasa, and Yoshinao Kobayashi performed

statistical analysis. Yasuyuki Tamai, Ryuta Shigefuku, Hiroshi Hasegawa, Masatoshi Watanabe, Hayato Nakagawa, Yuki Matsushita, Takuma Nakatsuka, Kenichiro Enooku, and Hayato Nakagawa were involved in data interpretation and in technical and material support. Akiko Eguchi conceived the idea, designed experiments, assisted in data analyses and in drafting and critical review of the manuscript, and provided funding for the study. All authors critically revised the manuscript for important intellectual content.

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