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ORIGINAL ARTICLE

Limited applicability of cathepsin D for the diagnosis and monitoring of non-alcoholic steatohepatitis

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Abstract

Background and Aim: To date, there are limited data on the applicability of cathepsin D for the diagnosis and monitoring of non-alcoholic steatohepatitis (NASH).
Methods: This study included patients with biopsy-proven non-alcoholic fatty liver disease (NAFLD) diagnosed between November 2012 and October 2015. Serum cathepsin D levels were measured using the CatD enzyme-linked immunosorbent assay (USCN Life Science, Wuhan, China) using stored samples collected on the same day of the liver biopsy procedure. The performance of cathepsin D in the diagnosis and monitoring of NASH was evaluated using receiver operating characteristic analysis.
Results: Data for 216 liver biopsies and 34 healthy controls were analyzed. The mean cathepsin D level was not significantly different between NAFLD patients and controls; between NASH and non-NASH patients; and across the different steatosis, lobular inflammation, and hepatocyte ballooning grades. The area under receiver operating characteristic curve (AUROC) of cathepsin D for the diagnosis of NAFLD and NASH was 0.62 and 0.52, respectively. The AUROC of cathepsin D for the diagnosis of the different steatosis, lobular inflammation, and hepatocyte ballooning grades ranged from 0.51 to 0.58. Of the 216 liver biopsies, 152 were paired liver biopsies from 76 patients who had a repeat liver biopsy after 48 weeks. There was no significant change in the cathepsin D level at follow-up compared to baseline in patients who had histological improvement or worsening for steatosis, lobular inflammation, and hepatocyte ballooning grades. Cathepsin D was poor for predicting improvement or worsening of steatosis and hepatocyte ballooning, with AUROC ranging from 0.47 to 0.54. It was fair for predicting worsening (AUROC 0.73) but poor for predicting improvement (AUROC 0.54) of lobular inflammation.
Conclusion: Cathepsin D was a poor biomarker for the diagnosis and monitoring of NASH in our cohort of Asian patients, somewhat inconsistent with previous observations in Caucasian patients. Further studies in different cohorts are needed to verify our observation.

Introduction

In recent years, the prevalence of non-alcoholic fatty liver disease (NAFLD) has risen, along with the increasing prevalence of obesity, becoming the most common cause of chronic liver disease worldwide. It is estimated to affect approximately 25% of the general population.[1] NAFLD can be attributed to overnutrition and is strongly associated with obesity, insulin resistance, glucose intolerance, atherogenic dyslipidemia, and arterial hypertension.[2] It is considered the liver manifestation of the metabolic syndrome.[3] While NAFLD patients are at increased risk of cardiovascular disease in general, it is those patients with non-alcoholic steatohepatitis (NASH) who are at increased risk of liver-related complications and mortality, especially in the presence of advanced liver fibrosis.[4] NASH is characterized by lobular inflammation and hepatocyte ballooning, in addition to the excessive accumulation of fat, and is the more severe form of the disease that can lead to fibrosis, cirrhosis, liver failure, and liver cancer.

Currently, the diagnosis of NASH can only be made reliably with histopathological examination of a liver biopsy

specimen. However, the liver biopsy procedure is invasive, poorly accepted, and carries a small risk of serious complications.[5,6] A simple and reliable noninvasive test for the diagnosis and follow-up of NASH is much needed in the management of NAFLD patients. While elevated serum aminotransferase level, for example, aspartate aminotransferase level, has been shown to have good positive predictive value for the diagnosis of NASH,[7] it is well recognized that serum aminotransferase level may be normal even in the presence of advanced liver disease in NAFLD patients.[8] Our group has previously evaluated other biomarkers, namely, the cytokeratin-18 fragment CK18Asp396 or M30[9] and the *Wisteria floribunda* agglutinin-positive Mac-2 binding protein,[10] for the assessment of the severity of NAFLD using samples collected from our large cohort of biopsy-proven NAFLD patients. However, the search remained elusive.

Proteins that are involved in the pathophysiology of NASH may be utilized as potential biomarkers. One of the proposed mechanisms for the switch from hepatic steatosis to inflammation in NASH is autophagic dysfunction as a result of cholesterol accumulation in resident immune cells in the liver, such as the Kupffer cells. It has been postulated that cathepsins are implicated in this process. Recently, two studies published by the same group demonstrated that cathepsin D may be useful for the assessment of disease severity in NAFLD patients.[11,12] However, these results have not been validated by other groups and in an Asian cohort. Hence, we conducted this study with the aim of evaluating the performance of cathepsin D for the diagnosis and follow-up of NASH and its histological components in our cohort of Asian biopsy-proven NAFLD patients.

Methods

This study was approved by the University of Malaya Medical Centre's Medical Ethics Committee (approval no.: 201401–0660) and conformed to the Declaration of Helsinki. All subjects provided written informed consent. This study included all NAFLD patients who underwent a liver biopsy at the University of Malaya Medical Centre between November 2012 and October 2015. The included patients were those screened for a clinical trial on silymarin compared with placebo and those who had a repeat liver biopsy following 48 weeks of intervention in the same clinical trial. Detailed information on the aforementioned clinical trial can be found elsewhere.[13] The diagnosis of NAFLD was made following exclusion of significant alcohol intake, use of medications that can cause hepatic steatosis, viral hepatitis B and C infections, and other causes of chronic liver disease where indicated.[14] Demographic, clinical, anthropometric, and laboratory data were collected using standard protocol. Weight and height were measured using standard equipment. Obesity was defined as body mass index (BMI) ≥ 25 kg per m^2.[15] Waist circumference was measured at the mid-point between the lowest margin of the least palpable rib and the top of the iliac crest in the standing position. Central obesity was defined as waist circumference > 90 cm for men and > 80 cm for women.[16] Venous blood was drawn on the same day of the liver biopsy procedure after an overnight fast to assess complete blood count, blood glucose, glycated hemoglobin (HbA1c), lipid profile, and liver profile and for the measurement of serum M30 and cathepsin D levels. Biochemical measurements were performed using standard laboratory procedures.

The study planned for at least 30 controls recruited from persons attending the Endoscopy Unit, University of Malaya Medical Centre, for the investigation of dyspepsia or screening colonoscopy. All controls had no known medical illness and had an ultrasound examination to exclude fatty liver. Liver biopsy was not performed for controls. BMI and waist circumference were determined as described above. Venous blood was drawn after an overnight fast for liver profile and for the measurement of serum M30 and cathepsin D levels.

Liver biopsy and histological assessment. Ultrasonography-guided percutaneous liver biopsy was performed by either one of two experienced operators (Wah-Kheong Chan, Sanjiv Mahadeva) using an 18 G Temno II semiautomatic biopsy needle (Cardinal Health, Dublin, OH, USA). Liver biopsy specimens were processed using standard laboratory procedures. Liver biopsy slides were stained with hematoxylin and eosin stain and masson trichrome stain. They were then examined by an experienced histopathologist (Nik Raihan Nik Mustapha) who was blinded to clinical data. NASH was diagnosed based on the presence of steatosis, lobular inflammation, and ballooning (≥grade 1 each). Histopathological findings were reported according to the NASH Clinical Research Network Scoring System.[17] Fibrosis was staged 0–4 (F0 = no fibrosis, F1 = perisinusoidal or portal/periportal fibrosis, F2 = perisinusoidal and portal/periportal fibrosis, F3 = bridging fibrosis, F4 = cirrhosis). Advanced fibrosis was defined as fibrosis stage ≥F3.

Human cathepsin D enzyme-linked immunosorbent assay. The blood sample for measurement of serum cathepsin D level was collected in a plain tube on the same day of the liver biopsy procedure. It was processed to serum and stored at −80°C until further analysis. The serum was used for the quantitative measurement of cathepsin D through the CatD enzyme-linked immunosorbent assay according to the manufacturer's protocol (USCN Life Science, Wuhan, China). This assay has intra- and interassay coefficients of variability of <8 and <10%, respectively, and a lower limit of detection of 78 pg/mL, as per the kit insert. The characteristics of the assay from our evaluation are within the manufacturer's claim. The test was performed for all samples in a single session by a single investigator (Pavai Sthaneshwar) who was blinded to clinical data. Similarly, the serum was used for the quantitative measurement of M30 using the M30-Apoptosense ELISA kit (PEVIVA, Bromma, Sweden). This assay has intra- and interassay coefficients of variability of <10% and a lower limit of detection of 25 U/L, as per the kit insert. The characteristics of the assay from our evaluation are within the manufacturer's claim.

Statistical analysis. Data analysis was undertaken using R Foundation Statistical software (R 3.2.1, R Foundation for Statistical Computing, Vienna, Austria).[18,19] Continuous variables were expressed as mean ± SD or median (interquartile range) and analyzed using the *t*-test or Mann–Whitney test where appropriate. Categorical variables were expressed as percentages and analyzed using χ^2 test or Fisher's exact test where appropriate. Univariate analysis was performed to determine factors

associated with serum cathepsin D level. Factors that were significant on univariate analysis were included in multivariate analysis. Boxplots were used to illustrate serum cathepsin D, M30, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transpeptidase (GGT) levels for controls and NAFLD patients, NASH, and non-NASH patients and the different grades of steatosis, lobular inflammation, and hepatocyte ballooning. The area under receiver operating characteristic curve (AUROC) was calculated for serum cathepsin D, M30, ALT, AST, and GGT for the diagnosis of NAFLD; NASH; and the different grades of steatosis, lobular inflammation, and hepatocyte ballooning. AUROC was interpreted as follows: 0.90–1.00 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair, and <0.70 = poor. Optimal cut-off values were the values that provided the greatest sum of sensitivity and specificity. AUROC was also calculated for changes in serum cathepsin D, M30, ALT, AST, and GGT following changes in the grade of steatosis, lobular inflammation, and hepatocyte ballooning. For all analyses, a *P*-value of <0.05 was considered statistically significant.

Results

Patient characteristics. The data of 216 liver biopsies and 34 healthy controls were analyzed. The characteristics of the NAFLD patients and controls are presented in Table 1. The NAFLD patients were significantly older and consisted of a higher proportion of males compared with the controls. The NAFLD patients had significantly higher BMI and waist circumference and were more likely to have obesity and central obesity. The percentage of NAFLD patients with non-NASH and NASH was 28% (60/216) and 72% (160/216), respectively. The characteristics of non-NASH and NASH patients are presented in Table 2. The NASH patients were significantly older; had greater BMI and waist circumference; and were more likely to have central obesity, type 2 diabetes mellitus, and hypertension compared with non-NASH patients.

Table 1 Characteristics of NAFLD patients and healthy controls

	Healthy controls, *n* = 34	NAFLD patients, *n* = 216	*P*-value
Age (years)	32.4 ± 14.2	49.9 ± 11.4	<0.001
Male, *n* (%)	6 (18)	113 (52.3)	<0.001
Ethnicity, *n* (%)			0.092
Chinese	17 (50)	63 (29.2)	
Indian	3 (9)	39 (18.1)	
Malay	14 (41)	112 (51.9)	
Others	0 (0)	2 (0.9)	
BMI (kg per m^2)	21.8 ± 3.1	30.0 ± 4.4	<0.001
Obesity, *n* (%)	5 (15)	191 (88.4)	<0.001
Waist circumference (cm)	75 ± 10	99 ± 10	<0.001
Central obesity, *n* (%)	7 (20.6)	205 (94.9)	<0.001
ALT (U/L)	26 (22–32)	66 (43–103)	<0.001
AST (U/L)	20 (18–27)	40 (29–62)	<0.001
GGT (U/L)	31 (22–45)	79 (41–123)	<0.001
Cathepsin D (pg/mL)	226 (144–460)	328 (213–508)	0.030
M30 (U/L)	128 (103–168)	352 (229–614)	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma glutamyl transpeptidase; NAFLD, non-alcoholic fatty liver disease.

Cathepsin D, M30, ALT, AST, and GGT levels in NAFLD patients and controls. The mean cathepsin D, M30, ALT, AST, and GGT levels were significantly higher in NAFLD patients compared with controls (Table 1). Boxplots illustrating the cathepsin D, M30, ALT, AST, and GGT levels in NAFLD patients and controls can be found in Figure S1, (available online). The AUROC, optimal cut-off, sensitivity, specificity, positive predictive value, and negative predictive value of cathepsin D, M30, ALT, AST, and GGT for the diagnosis of NAFLD are shown in Table 3. M30, ALT, AST, and GGT were good to excellent, while cathepsin D was poor, for the diagnosis of NAFLD. The results of an additional analysis on 14 controls well matched in age and gender to the NAFLD patients that yielded similar results can be found in Tables S1 and S2 and Figure S2 (available online).

Cathepsin D, M30, ALT, AST, and GGT levels in non-NASH and NASH patients. The mean cathepsin D level was not significantly different between NASH patients and non-NASH patients. The mean M30, ALT, AST, and GGT levels were significantly higher in NASH patients compared with non-NASH patients (Table 2). Boxplots illustrating the cathepsin D, M30, ALT, AST, and GGT levels in non-NASH and NASH patients can be found in Figure S3 (available online). The AUROC, optimal cut-off, sensitivity, specificity, positive predictive value, and negative predictive value of cathepsin D, M30, ALT, AST, and GGT for the diagnosis of NASH are shown in Table 4. M30 and AST were fair, while cathepsin D, ALT, and GGT were poor, for the diagnosis of NASH. Serum cathepsin D level was associated with obesity, hypertension, dyslipidemia, and histological fibrosis stage on univariate analysis. On multivariate analysis, only dyslipidemia was significantly associated with serum cathepsin D level (Table 5).

Cathepsin D, M30, ALT, AST, and GGT levels according to steatosis, lobular inflammation, and hepatocyte ballooning grades. The cathepsin D, M30, ALT, AST, and GGT levels according to steatosis, lobular inflammation, and hepatocyte ballooning grades are presented in Figures S4–S6 (available online). Only ALT levels were significantly different across the steatosis grades. M30, ALT, AST, and GGT levels were significantly different across the lobular inflammation and hepatocyte ballooning grades. Cathepsin D levels were not significantly different across the steatosis, lobular inflammation, and hepatocyte ballooning grades. The AUROC, optimal cut-off, sensitivity, specificity, positive predictive value, and negative predictive value of cathepsin D, M30, ALT, AST, and GGT for the diagnosis of the different grades of steatosis, lobular inflammation, and hepatocyte ballooning are shown in Tables S3–S5 (available online). Cathepsin D, M30, ALT, AST, and GGT were poor for the diagnosis of the different steatosis grades. AST was the best predictor for the diagnosis of the different lobular inflammation grades and for the diagnosis of

Table 2 Characteristics of NASH and non-NASH patients

	NASH, $n = 156$	Non-NASH, $n = 60$	*P*-value
Age (years)	50.9 ± 11.4	47.3 ± 10.9	0.035
Male, *n* (%)	75 (48)	38 (63)	0.063
Ethnicity			0.810
Chinese	46 (29.5)	17 (28.3)	
Indian	27 (17.3)	12 (20.0)	
Malay	81 (51.9)	31 (51.7)	
Others	2 (1.3)	0 (0.0)	
BMI (kg per m^2)	30.5 ± 4.4	28.8 ± 4.1	0.010
Obesity, *n* (%)	140 (90)	51 (85)	0.460
Waist circumference (cm)	100 ± 10	96 ± 9	0.004
Central obesity, *n* (%)	153 (98)	52 (87)	0.002
Type 2 diabetes mellitus, *n* (%)	94 (60)	19 (32)	<0.001
Hypertension, *n* (%)	104 (67)	25 (42)	0.001
Dyslipidemia, *n* (%)	121 (78)	41 (68)	0.219
FBS (mmol/L)	6.00 (5.20–7.40)	5.50 (5.00–6.20)	0.015
HbA1c (%)	6.55 (5.70–7.60)	5.70 (5.32–6.07)	<0.001
Albumin (g/L)	43 (41–45)	44 (42–46)	0.034
ALT (U/L)	74 (48–112)	49 (36–72)	<0.001
AST (U/L)	50 (33–71)	31 (23–38)	<0.001
GGT (U/L)	83 (52–129)	45 (32–90)	<0.001
Platelet (× 10^9/L)	264 (226–303)	283 (254–322)	0.033
Triglyceride (mmol/L)	1.60 (1.29–2.00)	1.60 (1.30–2.00)	0.818
Total cholesterol (mmol/L)	4.70 (4.10–5.43)	5.10 (4.40–5.75)	0.021
HDL (mmol/L)	1.11 (0.97–1.30)	1.20 (1.00–1.39)	0.129
LDL (mmol/L)	2.75 (2.23–3.56)	3.03 (2.50–3.56)	0.059
Cathepsin D (pg/mL)	328 (206–482)	328 (244–525)	0.722
M30 (U/L)	433 (279–743)	253 (182–340)	<0.001
Biopsy length (mm)	15 (12–16)	14 (12–15)	0.312
Number of portal tracts	8 (7–10)	7 (5–9)	0.005
Steatosis grade (%)			0.356
S1	46 (30)	12 (20)	
S2	78 (50)	33 (55)	
S3	32 (20)	15 (25)	
Inflammation grade (%)			<0.001
0	0 (0)	4 (7)	
1	62 (40)	51 (85)	
2	88 (56)	5 (8)	
3	6 (4)	0 (0)	
Ballooning grade (%)			<0.001
0	0 (0)	57 (95)	
1	102 (65)	3 (5)	
2	54 (35)	0 (0)	
NAFLD activity score	5 (4–6)	3 (3–4)	<0.001
Fibrosis stage (%)			<0.001
F0	28 (18)	42 (70)	
F1	73 (47)	15 (25)	
F2	15 (10)	1 (2)	
F3	34 (22)	2 (3)	
F4	6 (4)	0 (0)	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; GGT, gamma glutamyl transpeptidase; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

hepatocyte ballooning grade 2. The AUROC of AST level for the diagnosis of lobular inflammation grade ≥2 and 3 was 0.81 and 0.91, respectively. The AUROC of AST level for the diagnosis of hepatocyte ballooning grade 2 was 0.80. M30 was the best predictor for the diagnosis of hepatocyte ballooning grade ≥ 1, with an AUROC of 0.72.

Changes in cathepsin D, M30, ALT, AST, and GGT levels according to changes in steatosis, lobular inflammation, and hepatocyte ballooning grades. Of the 216 liver biopsies, 152 were paired liver biopsies from 76 patients who had a repeat liver biopsy after 48 weeks of intervention. The mean cathepsin D, M30, ALT, AST, and GGT levels at baseline and follow-up according to changes in steatosis, lobular inflammation, and hepatocyte ballooning are shown in Tables 6–8. The changes in cathepsin D, M30, ALT, AST, and GGT levels according to changes in steatosis, lobular inflammation, and hepatocyte ballooning grades are shown in Figures S7–S9 (available online). Patients who had improvement in steatosis had a significant reduction in ALT, AST, and GGT levels. The mean changes in ALT, AST, and GGT levels in patients with improvement in steatosis were significant when compared with patients who had no change in steatosis. Patients who had improvement in inflammation had significant reductions in M30, ALT, AST, and GGT. The mean changes in ALT and AST levels in patients with improvement in inflammation were significant when compared with patients who had no change in inflammation and when compared with patients who had worsened inflammation. In addition, the mean change in ALT level in patients with worsened inflammation was significant when compared with patients who had no change in inflammation. The mean change in cathepsin D levels in patients with worsening inflammation was significant compared to patients with no change in inflammation. In contrast, patients who had improvement in ballooning had significant reductions in M30, ALT, AST, and GGT. The mean changes in cathepsin D, ALT, AST, and GGT in patients with improvement in ballooning were significant when compared to worsened ballooning. In addition, the mean change in GGT level in patients with improvement in ballooning was significant when compared with patients who had no change in ballooning. The AUROC, optimal cut-off, sensitivity, specificity, positive predictive value, and negative predictive value of change in cathepsin D, M30, ALT, AST, and GGT for predicting change in grades of steatosis, lobular inflammation,

Table 3 Accuracy of serum cathepsin D, M30 ALT, AST, and GGT for identification of NAFLD

	AUROC	Optimal cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Cathepsin D	0.62 (0.50–0.73)	250 pg/mL	70	56	91	23	68
M30	0.91 (0.86–0.95)	177 U/L	89	79	97	54	88
AST	0.90 (0.85–0.96)	31 U/L	71	100	100	19	73
ALT	0.93 (0.89–0.97)	35 U/L	89	93	99	37	89
GGT	0.86 (0.79–0.93)	55 U/L	65	100	100	16	67

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under receiver operating characteristic curve; GGT, gamma glutamyl transpeptidase; NAFLD, non-alcoholic fatty liver disease; NPV, negative predictive value; PPV, positive predictive value.

Table 4 Accuracy of serum cathepsin D, M30, ALT, AST, and GGT for identification of NASH

	AUROC	Optimal cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Cathepsin D	0.52 (0.43–0.60)	218 pg/mL	82	28	30	80	43
M30	0.73 (0.65–0.80)	346 U/L	78	63	45	88	67
AST	0.75 (0.68–0.82)	47 U/L	87	53	42	91	63
ALT	0.69 (0.61–0.77)	61 U/L	67	63	41	83	64
GGT	0.67 (0.58–0.76)	46 U/L	52	81	52	81	73

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under receiver operating characteristic curve; GGT, gamma glutamyl transpeptidase; NASH, non-alcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value.

Table 5 Univariate and multivariate analysis of factors associated with serum cathepsin D level

	Univariate analysis		Multivariate analysis	
	β	*P*	β	*P*
Obesity	−0.204	0.044	−0.168	0.097
Hypertension	−0.138	0.042	−0.096	0.349
Dyslipidemia	0.148	0.030	0.205	0.045
Fibrosis stage	−0.138	0.043	−0.107	0.298

Table 6 Baseline and follow-up serum cathepsin D, M30, ALT, AST, and GGT levels according to changes in steatosis

	Baseline	Follow-up	*P*-value
Improvement			
Cathepsin D (pg/mL)	287 (209–390)	318 (181–438)	0.872
M30 (U/L)	392 (270–805)	276 (211–361)	0.079
ALT (U/L)	88 (60–117)	43 (36–63)	<0.001
AST (U/L)	53 (35–73)	34 (26–40)	0.002
GGT (U/L)	103 (82–134)	71 (37–94)	0.009
No change			
Cathepsin D (pg/mL)	352 (261–522)	354 (176–548)	0.703
M30 (U/L)	466 (250–670)	373 (210–784)	1.000
ALT (U/L)	74 (56–123)	61 (41–102)	0.071
AST (U/L)	47 (31–69)	49 (30–62)	0.621
GGT (U/L)	87 (54–148)	76 (40–128)	0.402
Worsening			
Cathepsin D (pg/mL)	260 (185–433)	246 (126–498)	0.862
M30 (U/L)	349 (323–594)	395 (356–771)	0.418
ALT (U/L)	80 (62–133)	81 (62–94)	0.487
AST (U/L)	59 (38–80)	51 (43–82)	0.685
GGT (U/L)	82 (48–96)	66 (50–89)	0.487

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase.

and hepatocyte ballooning are shown in Tables S6–S8. Cathepsin D was a poor marker for identifying changes in steatosis, inflammation, and ballooning except for worsening of inflammation, for which it performed fairly. Changes in ALT, AST, and GGT were fair at identifying improvement of steatosis. Change in ALT was good, while changes in cathepsin D, AST, and GGT were fair, in identifying worsened inflammation. Change in AST was also fair in identifying improvement in inflammation. M30 was fair for identifying improvement and good for identifying worsening of ballooning. ALT and AST were also fair for identifying worsening of ballooning.

Discussion

Our study evaluated cathepsin D as a noninvasive biomarker in a large cohort of Asian biopsy-proven NAFLD patients and found that it was poor for the diagnosis of NAFLD, NASH, steatosis, lobular inflammation, and hepatocyte ballooning when compared with other biomarkers, such as M30, ALT, AST, and GGT. This study also demonstrated cathepsin D to be poor in predicting changes in steatosis, lobular inflammation, and hepatocyte ballooning using paired biopsy.

The cathepsin family comprises the catalytic serine (cathepsin G), aspartate (cathepsins D and E), and cysteine (cathepsins B, C, H, F, K, L, O, S, V, and W) peptidases that exhibit endopeptidase or exopeptidase activities.[20–24] Apart from their known role in protein turnover, recent data have demonstrated that cathepsins are also involved in liver injury and fibrogenesis. For instance, cathepsins T and D activation has been observed in experimental fibrogenesis,[25] and previous studies reported high levels of cathepsins B, D, and L in the serum of patients with cirrhosis and hepatocellular carcinoma.[26–28] Apart from inflammation, cathepsin D is thought to play a role in the progression of fibrosis from in vitro studies, demonstrating its role in hepatic stellate cell-mediated fibrogenesis in the liver. Moles *et al.* demonstrated that levels of cathepsin D were significantly increased

Table 7 Baseline and follow-up serum cathepsin D, M30, ALT, AST, and GGT levels according to changes in inflammation

	Baseline	Follow-up	*P*-value
Improvement			
Cathepsin D (pg/mL)	342 (206–496)	214 (117–523)	0.544
M30 (U/L)	518 (279–796)	274 (178–440)	0.039
ALT (U/L)	80 (61–125)	55 (35–72)	<0.001
AST (U/L)	52 (35–96)	40 (28–53)	0.002
GGT (U/L)	84 (52–116)	51 (34–81)	0.003
No change			
Cathepsin D (pg/mL)	314 (216–434)	322 (176–518)	0.277
M30 (U/L)	431 (239–606)	345 (222–702)	0.482
ALT (U/L)	74 (53–120)	56 (40–96)	0.224
AST (U/L)	43 (32–67)	39 (29–60)	0.142
GGT (U/L)	92 (67–139)	74 (51–125)	0.655
Worsening			
Cathepsin D (pg/mL)	415 (356–1135)	256 (181–543)	0.671
M30 (IU/L)	288 (284–708)	372 (235–564)	0.783
ALT (U/L)	73 (60–97)	111 (102–142)	0.067
AST (U/L)	42 (37–62)	67 (53–80)	0.644
GGT (U/L)	124 (69–149)	91 (79–162)	0.468

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase.

during in vitro mouse hepatic stellate cell activation and that this upregulation occurred largely in lysosomes, although a significant fraction is secreted in the extracellular media.[29]

To date, there are limited clinical data on the usefulness of cathepsin D as a noninvasive marker in NAFLD patients. Walenbergh *et al*. were the first to report on the potential use of cathepsin D as a noninvasive marker in pediatric NAFLD patients. In their study on 96 children with biopsy-proven NAFLD, they found cathepsin D to have a high diagnostic accuracy for the differentiation between steatosis and hepatic inflammation, with an AUROC of 0.94. However, the high diagnostic accuracy was yielded following exclusion of patients with borderline NASH. When patients with borderline NASH were included, the diagnostic accuracy was 0.81–0.88. This is still much better than the AUROC of 0.50 that we found in our study on adult patients. One possible explanation is the presence of other conditions in adult patients that may have affected the level and accuracy of cathepsin D for the diagnosis of NASH, rendering it less useful compared with that in the pediatric population.

Table 8 Baseline and follow-up serum cathepsin D, M30, ALT, AST, and GGT levels according to changes in ballooning

	Baseline	Follow-up	*P*-value
Improvement			
Cathepsin D (pg/mL)	312 (200–473)	280 (73–503)	0.763
M30 (U/L)	484 (280–653)	251 (171–329)	0.003
ALT (U/L)	78 (56–110)	44 (35–70)	<0.001
AST (U/L)	48 (32–65)	32 (26–45)	0.004
GGT (U/L)	84 (54–134)	52 (34–82)	0.006
No change			
Cathepsin D (pg/mL)	354 (268–487)	354 (176–608)	0.958
M30 (U/L)	340 (220–755)	388 (279–616)	0.115
ALT (U/L)	74 (59–128)	64 (43–104)	0.529
AST (U/L)	51 (34–78)	50 (35–64)	0.227
GGT (U/L)	92 (56–130)	74 (52–160)	0.401
Worsening			
Cathepsin D (pg/mL)	270 (176–368)	256 (194–312)	0.942
M30 (U/L)	461 (432–670)	1099 (647–1324)	0.344
ALT (U/L)	73 (49–109)	103 (96–110)	0.084
AST (U/L)	52 (42–64)	62 (55–81)	0.397
GGT (U/L)	129 (82–178)	102 (78–189)	0.412

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase.

It is interesting to note that, when the same group of investigators (Walenbergh *et al*.) studied adult subjects with NAFLD, cathepsin D levels were found to be increased in NASH. This was contrary to their initial published study on pediatric NAFLD patients, where cathepsin D levels were found to be reduced in NASH patients. The investigators cited differences in the pathophysiology of NASH in children and adults for the observed inconsistency. However, despite overall higher levels in NASH subjects compared to controls, cathepsin D levels actually decreased with increasing severity of NASH in that study. Contrary to the work by Walenbergh *et al*., we found that cathepsin D level could not differentiate between NASH and non-NASH patients. This may be due to the larger proportion of NASH patients with more severe liver disease in our study. However, we did not observe significant differences in cathepsin D level according to the individual histological components of NASH, suggesting that other differences in the study populations may be contributory. For example, our study population included Asian patients with a lower BMI, while two of the three cohorts in the study by Walenbergh *et al*. included bariatric Caucasian patients.

Assuming that all NASH patients who had obesity surgery in the Kuopio cohort had liver histological improvement after 1 year, Walenbergh *et al*. also suggested that cathepsin D may be useful for the follow-up of NASH patients based on the observed significant decline in cathepsin D among the NASH patients. However, using paired liver biopsies, we found that cathepsin D was poor in predicting changes in hepatic steatosis, lobular inflammation, and hepatocyte ballooning. In view of these findings, we are of the opinion that cathepsin D is not a useful test for diagnosis and monitoring of NASH. In view of the conflicting results, further studies in different populations are needed to clarify the role of cathepsin D in the assessment and follow-up of NAFLD patients.

Our study is the only study to date that evaluated the performance of cathepsin D in Asian NAFLD patients and represents the only other study on adult NAFLD patients on this matter. With 216 liver biopsies and 34 healthy controls, it is also the largest study to date. Moreover, the availability of paired liver biopsy allowed the evaluation of cathepsin D for follow-up of NAFLD patients. The study was carried out prospectively, and the collection of blood samples was performed on the same day as the liver biopsy procedure to minimize differences due to changes over time. Despite our best effort, the study had several limitations. First, study subjects who had a liver biopsy for assessment of the severity of their NAFLD were recruited from a tertiary care hospital and may have more severe liver disease compared with NAFLD patients in the general population. Thus,

the findings of this study may not be generalizable to NAFLD patients in the general population. Second, as in any study using the histopathological examination of liver biopsy specimen as a reference, there may have been sampling and observer variability. Third, the absence of NAFLD in controls was based on ultrasonography, which may lack sensitivity in the detection of mild hepatic steatosis. However, performing a liver biopsy on healthy controls would not be acceptable.

In conclusion, cathepsin D is a poor biomarker for evaluating the diagnosis or changes in disease activity of NAFLD in our cohort of Asian patients, somewhat inconsistent with previous observations in Caucasian patients. In this modern era, where there is increasing interest in the use of noninvasive biomarkers in NAFLD, our findings will add to the limited literature on the usefulness (or lack of) of cathepsin D. Future studies should aim to confirm our findings in different cohorts of patients with biopsy-proven NAFLD.

References

1 Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016; **64**: 73–84.

2 Wong VW, Chan WK, Chitturi S *et al*. The Asia-Pacific Working Party on nonalcoholic fatty liver disease guidelines 2017 Part 1: definition, risk factors and assessment. *J. Gastroenterol. Hepatol.* 2018; **33**: 70–85.

3 Day CP. From fat to inflammation. *Gastroenterology*. 2006; **130**: 207–10.

4 Musso G, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann. Med.* 2011; **43**: 617–49.

5 Mahadeva S, Mahfudz AS, Vijayananthan A. Ethnicity influences pain after ultrasound-guided percutaneous liver biopsy. *Eur. J. Gastroenterol. Hepatol.* 2015; **27**: 1378–81.

6 Mueller M, Kratzer W, Oeztuerk S *et al*. Percutaneous ultrasonographically guided liver punctures: an analysis of 1961 patients over a period of ten years. *BMC Gastroenterol.* 2012; **12**: 173.

7 Loo SY, Chan WK. Emerging new standard for non-invasive assessment of liver disease mortality in non-alcoholic fatty liver disease. *Hepatobiliary Surg. Nutr.* 2017; **6**: 135–7.

8 Wong VW, Wong GL, Tsang SW *et al*. Metabolic and histological features of non-alcoholic fatty liver disease patients with different serum alanine aminotransferase levels. *Aliment. Pharmacol. Ther.* 2009; **29**: 387–96.

9 Chan WK, Sthaneshwar P, Nik Mustapha NR, Mahadeva S. Limited utility of plasma M30 in discriminating non-alcoholic steatohepatitis from steatosis–a comparison with routine biochemical markers. *PLoS One*. 2014; **9**: e105903.

10 Lai LL, Chan WK, Sthaneshwar P, Nik Mustapha NR, Goh KL, Mahadeva S. Serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein in non-alcoholic fatty liver disease. *PLoS One*. 2017; **12**: e0174982.

11 Walenbergh SM, Houben T, Hendrikx T *et al*. Plasma cathepsin D levels: a novel tool to predict pediatric hepatic inflammation. *Am. J. Gastroenterol.* 2015; **110**: 462–70.

12 Walenbergh SM, Houben T, Rensen SS *et al*. Plasma cathepsin D correlates with histological classifications of fatty liver disease in adults and responds to intervention. *Sci. Rep.* 2016; **6**: 38278.

13 Wah Kheong C, Nik Mustapha NR, Mahadeva S. A randomized trial of silymarin for the treatment of nonalcoholic steatohepatitis. *Clin. Gastroenterol. Hepatol.* 2017; **15**: 1940–1949.e1948.

14 Chalasani N, Younossi Z, Lavine JE *et al*. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology*. 2012; **55**: 2005–23.

15 Anuurad E, Shiwaku K, Nogi A *et al*. The new BMI criteria for asians by the regional office for the western pacific region of WHO are suitable for screening of overweight to prevent metabolic syndrome in elder Japanese workers. *J. Occup. Health*. 2003; **45**: 335–43.

16 Alberti KG, Zimmet P, Shaw J. The metabolic syndrome–a new worldwide definition. *Lancet*. 2005; **366**: 1059–62.

17 Kleiner DE, Brunt EM, Van Natta M *et al*. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; **41**: 1313–21.

18 R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2018. URL https://www.R-project.org/.

19 Robin X, Turck N, Hainard A *et al*. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011: **12**: 77.

20 Koike M, Shibata M, Ohsawa Y *et al*. Involvement of two different cell death pathways in retinal atrophy of cathepsin D-deficient mice. *Mol. Cell. Neurosci.* 2003; **22**: 146–61.

21 Nakagawa T, Roth W, Wong P *et al*. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science*. 1998; **280**: 450–3.

22 Nakanishi H, Zhang J, Koike M *et al*. Involvement of nitric oxide released from microglia-macrophages in pathological changes of cathepsin D-deficient mice. *J. Neurosci.* 2001; **21**: 7526–33.

23 Saftig P, Hetman M, Schmahl W *et al*. Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. *EMBO J.* 1995; **14**: 3599–608.

24 Urbich C, Heeschen C, Aicher A *et al*. Cathepsin L is required for endothelial progenitor cell-induced neovascularization. *Nat. Med.* 2005; **11**: 206–13.

25 Gohda E, Nagahama J, Nakamura O, Tsubouchi H, Daikuhara Y, Pitot HC. Increased activities of liver cathepsins T and D in carbon tetrachloride-treated rats. *Biochim. Biophys. Acta*. 1984; **802**: 362–71.

26 Leto G, Tumminello FM, Pizzolanti G *et al*. Cathepsin D serum mass concentrations in patients with hepatocellular carcinoma and/or liver cirrhosis. *Eur. J. Clin. Chem. Clin. Biochem.* 1996; **34**: 555–60.

27 Leto G, Tumminello FM, Pizzolanti G, Montalto G, Soresi M, Gebbia N. Lysosomal cathepsins B and L and Stefin A blood levels in patients with hepatocellular carcinoma and/or liver cirrhosis: potential clinical implications. *Oncology*. 1997; **54**: 79–83.

28 Yamamoto H, Murawaki Y, Kawasaki H. Collagenolytic cathepsin B and L activity in experimental fibrotic liver and human liver. *Res. Commun. Chem. Pathol. Pharmacol.* 1992; **76**: 95–112.

29 Moles A, Tarrats N, Fernández-Checa JC, Marí M. Cathepsins B and D drive hepatic stellate cell proliferation and promote their fibrogenic potential. *Hepatology*. 2009; **49**: 1297–307.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1 Cathepsin D, M30, ALT, AST, and GGT levels in NAFLD patients and controls.

Figure S2 Cathepsin D, M30, ALT, AST, and GGT levels in NAFLD patients and 14 age- and gender-matched healthy controls.
Figure S3 Cathepsin D, M30, ALT, AST, and GGT levels in non-NASH and NASH patients.
Figure S4 Cathepsin D, M30, ALT, AST, and GGT levels according to steatosis grades.
Figure S5 Cathepsin D, M30, ALT, AST, and GGT according to lobular inflammation grades.
Figure S6 Cathepsin D, M30, ALT, AST, and GGT according to hepatocyte ballooning grades.
Figure S7 Mean change in cathepsin D, M30, ALT, AST, and GGT according to changes in steatosis grade.
Figure S8 Mean change in cathepsin D, M30, ALT, AST, and GGT according to changes in lobular inflammation.
Figure S9 Mean change in cathepsin D, M30, ALT, AST, and GGT according to changes in hepatocyte ballooning.
Table S1 Characteristic of NAFLD patients compared with 14 age- and gender-matched healthy controls.
Table S2 Accuracy of serum cathepsin D, M30 ALT, AST, and GGT for identification of NAFLD (compared with 14 age- and gender-matched healthy controls).
Table S3 Accuracy of serum cathepsin D, M30, ALT, AST, and GGT for identification of different steatosis grades.
Table S4 Accuracy of serum cathepsin D, M30, ALT, AST and GGT for identification of different inflammation grades.
Table S5 Accuracy of serum cathepsin D, M30, ALT, AST, and GGT for identification of different ballooning grades.
Table S6 Accuracy of changes of serum cathepsin D, M30, ALT, AST, and GGT for identification of changes to steatosis grades.
Table S7 Accuracy of changes of serum cathepsin D, M30, ALT, AST, and GGT for identification of changes to inflammation grades.
Table S8 Accuracy of changes of serum cathepsin D, M30, ALT, AST, and GGT for identification of changes to ballooning grades.