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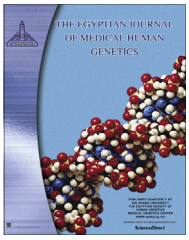

ORIGINAL ARTICLE

Cytokeratin 18 as a non invasive marker in diagnosis of NASH and its usefulness in correlation with disease severity in Egyptian patients

Mohsen M. Maher [a], Wesam A. Ibrahim [a], Shereen A. Saleh [a,*], Lobna Shash [b], Hoda Abou Gabal [b], Mohammed Tarif [c], Mohammed Gamal [a]

[a] *Department of General Internal Medicine, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[b] *Department of Surgical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt*
[c] *Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt*

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KEYWORDS

Cytokeratin 18;
NASH;
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NAFLD activity score;

Abstract *Background:* A simple noninvasive test that accurately distinguishes NASH from NAFL as well as determines the disease severity is urgently needed. Recently, it was found that determination of cytokeratin-18 (CK-18) fragments in the blood, predicts and correlates with histological NASH in which there is development of lobular inflammation, cell ballooning and fibrosis, supporting its usefulness in clinical practice.

Abbreviations: ALT, alanine transaminase; ANA, antinuclear antibody; Anti LKM Ab,anti-liver kidney microsomal antibodies; ASMA, anti smooth muscle antibody; AST, aspartate transaminase; BMI, body mass index; CBC, complete blood count; CK-18, cytokeratin-18; CK18-Asp396, caspase-cleaved cytokeratin 18; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; FBS, fasting blood sugar; HA1C, glycated hemoglobin; HBcAb, hepatitis B core antibody; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV Ab, hepatitis c antibody; HS, highly significant; IBM, *International Business Machines*; IR, insulin resistance; LDL, low density lipoprotein; mRNA, messenger ribonucleic acid; NAFL, non alcoholic fatty liver; NAFLD, non alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non alcoholic steatohepatitis; NS, insignificant; PT, prothrombin time; ROC, receiver operating characteristic; S, significant; SD, standard deviation; SPSS, statistical package for special science; T2DM, type 2 diabetes mellitus; TPS ELISA, tryptase ELISA.

* Corresponding author at: 5th Settlement, 90th Road, Compound Lake View, 51/1, Cairo, Egypt. Tel.: +20 26131342 (home), mobile: +20 01227834104.
E-mail address: shereen_saleh2014@hotmail.com (S.A. Saleh).

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Liver biopsy;
Keratins

Aims: To evaluate the role of CK-18 as a non invasive marker in diagnosis of NASH and its usefulness in correlation with disease severity in Egyptian patients.

Patients and methods: 90 subjects were divided into 3 groups: group I: including 30 patients with NASH, group II: including 30 patients with NAFL, and group III: including 30 healthy subjects as control. Diagnosis of NASH and its discrimination from NAFL was done by liver biopsy. CK-18 level in plasma was measured for all subjects using ELISA.

Results: CK-18 was significantly elevated in patients of group I in comparison to group II and III patients, with mean ± SD: 460 ± 279, 167 ± 56 and 149 ± 57, respectively, and *P* value: 0.001. The (ROC) curve diagnostic performance of CK18 in diagnosis of NASH shows: cutoff value of >240 U/L, with sensitivity 76.7%, specificity 95.0%. Ck-18 was found to correlate with disease severity assessed by NAS scoring system with *P* value: 0.001.

Conclusion: Measurement of CK18 in NASH is a useful screening, diagnostic and staging biomarker.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of conditions associated with over accumulation of fat in the liver ranging from nonalcoholic fatty liver disease NAFLD to nonalcoholic steatohepatitis (NASH) and cirrhosis [1].

Although NAFL typically follows a benign non progressive clinical course, NASH is a potentially serious condition; as many as 25% of patients may progress to cirrhosis and experience complications of portal hypertension, liver failure, and hepato-cellular carcinoma [2].

It was suggested that progression from NAFL to NASH and to advanced fibrosis results from two distinct events; first, insulin resistance (IR) leading to the accumulation of fat within hepatocytes, and second, mitochondrial reactive oxygen species causing lipid peroxidation and cytokine induction [3].

Cytokeratins are proteins of keratin-containing intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue. In 2006 a new systematic nomenclature for keratins was created and now the proteins previously called "cytokeratins" are simply called keratins [4].

When hepatocytes are chronically exposed to oxidative stress and toxic substances, they become ballooned, accumulate fat, show a disruption in the keratin intermediate filament network, and form Mallory bodies [5]. A Mallory body is composed of abnormally phosphorylated and cross-linked keratins, such as cytokeratin (CK) 8 and 18 and stress-induced proteins [6].

Since hepatocytes containing Mallory bodies are susceptible to apoptosis, so those levels of Mallory body-associated proteins released from hepatocytes into peripheral blood may be increased in NASH patients and change in accordance with disease activity [7].

At present, liver biopsy remains the only reliable and the "gold standard" way of diagnosing NASH, grading the severity of liver damage and for assessing fibrosis and architectural alterations [8]. Liver biopsy is an invasive procedure that carries a risk of complications. It is an imperfect tool; due to sampling errors, biopsy size (5–30 mm) and intra- and inter-observer variability, it is now agreed that biopsy is an "imperfect Gold Standard diagnostic tool" [9]. Hence new simple and non-invasive test that both accurately distinguishes NASH from NAFLD and determines the stage and grade of the disease is urgently needed [10].

In the current study we investigated the role of serum cytokeratin 18 as a noninvasive maker in differentiating NASH from NAFL, and its correlation with the disease severity.

2. Patients and methods

This study was conducted on 90 Egyptian individuals selected from Internal Medicine and Hepatology outpatient clinics and inpatient wards of Ain Shams University Hospitals (from Dec 2012 to Jul 2013).

They were classified into 3 groups:

- *Group I:* included 30 patients with NASH diagnosed by abdominal ultrasonography and liver biopsy, with no history of consumption of alcohol or hepatotoxic drugs.
- *Group II:* included 30 patients with NAFL diagnosed by abdominal ultrasonography and liver biopsy with no consumption of alcohol or hepatotoxic drugs.
- *Group III:* included 30 age-matched healthy controls attending Blood Bank as blood donors or coming for pre-employment check up with normal abdominal ultrasonography, normal aminotransferases, and no history of chronic liver disease.

Inclusion criteria: patients with NASH and NAFLD on top of Diabetes Mellitus (DM), obesity diagnosed by laboratory tests, ultrasonography and liver biopsy.

Exclusion criteria: liver diseases other than fatty liver as: chronic viral hepatitis, autoimmune hepatitis, liver cirrhosis, hepatocellular carcinoma (HCC) patients and significant alcoholic consumption (more than 21 drinks/week in males and 14 drinks/week in females).

- A consent was taken from all individuals in this study. The study was approved by the ethical committee of Ain Shams University School of medicine. The work has been carried out in accordance with the code of Ethics of the Wold Medical Association (Declaration of Helsinki) for experiments in humans.

All patients and controls were subjected to the following:

- Complete history taking and full physical examination, including body mass index (BMI).
- Laboratory investigations including: complete blood count (CBC), fasting blood sugar (FBS), glycated hemoglobin (HA1C), Liver function tests: alanine transaminase (ALT), aspartate transaminase (AST), Serum bilirubin, serum albumin and prothrombin time (PT), lipid profile (cholesterol and triglycerides), Viral markers (HBsAg, HBcAb, HCV Ab), Serum ferritin, immunoglobulins, ANA, ASMA, anti LKM Ab and cytokeratin 18 level in plasma by ELISA.
- Abdominal ultrasonography.
- Liver biopsy for patients of groups I and II.
- Fibroscan for control subjects to exclude liver fibrosis.

2.1. Histopathological examination

Ultrasonography-guided liver biopsies were performed under conscious sedation using a 16-gauge Klatskin needle. The length of the histological specimens was no less than 2.5 cm. All biopsy specimens were placed in 10% neutral buffered formalin solution for fixation and embedded in paraffin blocks. Serial sections (sectioned at 4-μm intervals) were concurrently stained with Hematoxylin-Eosin and Masson's trichrome. An experienced pathologist blinded to the clinical data scored the liver biopsies according to the NAFLD activity score (NAS) in which:

1. *Steatosis* (S) was scored from 0 to 3 with a four-grade scoring system from S0 to S3. S0: no steatosis or less than 5%, S1: 5–33%, S2: > 33–66%, S3: > 66%).
2. *Lobular inflammation* was staged as: stage 0: no foci, stage 1: < 2 foci, stage 2: 2–4 foci, stage 3: > 4 foci per 200× field.
3. *Ballooning* was staged as: stage 0: none, stage 1: few, stage 2: many ballooned cells.
4. *Fibrosis* was staged as: stage 0 with no fibrosis, stage 1 as perisinusoidal or periportal fibrosis, stage 2 as perisinusoidal and portal/periportal fibrosis, stage 3 as bridging fibrosis, stage 4 as cirrhosis.

The histological NAS was defined as the sum of the scores for steatosis (0–3), lobular inflammation (0–3), cell ballooning (0–2), and fibrosis (0–4). An NAS < 3 corresponds to nonalcoholic fatty liver (NAFL), 3–4 corresponds to borderline nonalcoholic steatohepatitis (NASH), and a score ⩾5 corresponds to NASH [11].

2.2. Measurement of caspase-generated CK-18 fragments in the blood

For all the patients in our study, a blood sample was taken before liver biopsy. Blood samples were centrifuged at 2500×*g* for 10 min and serum aliquots were stored at −20 °C until immediately before assay. All samples were analyzed in duplicate and blinded as to clinical status. Serum levels of CK18-Asp396 were determined by commercially available immunoassays according to the manufacturer's protocol.

Concentrations of CK18-Asp396 were expressed in units per liter (U/L) according to the manufacturer's instructions. The minimal detectable concentration in TPS ELISA is 6 U/L. The assay has no "high-dose hook effect" up to 20,000 U/L. The working range is 20–800 U/L. The 95th percentile for apparently healthy individuals has been determined to 80 U/L.

3. Statistical analysis

Data was analyzed on an IBM personal computer, using statistical package for special science (SPSS) software computer program version 15.

- Data were described as mean ± Standard Deviation (SD) for quantitative variables and as frequency and percentage for qualitative variables. Independent Student *t* test was used for comparison of quantitative variables among two independent groups. Paired *t* test was used for comparison of quantitative variables among groups. Chi-square test was used for comparison of distribution of qualitative variables among different groups. Correlation between continuous variables was performed using Pearson correlation coefficient.
- *Significance level (P) value*:
  - o $P \leqslant 0.05$ is significant (S).
  - o $P < 0.01$ is highly significant (HS).
  - o $P > 0.05$ is insignificant (NS).

- *ROC curve* is a graphical plot which illustrates the performance as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the total actual positives (sensitivity) vs. the fraction of false positives out of the total actual negatives (specificity), at various threshold settings.

4. Results and discussion

Descriptive data of the studied groups:

- This study included 90 Egyptian subjects who were divided into 3 groups:

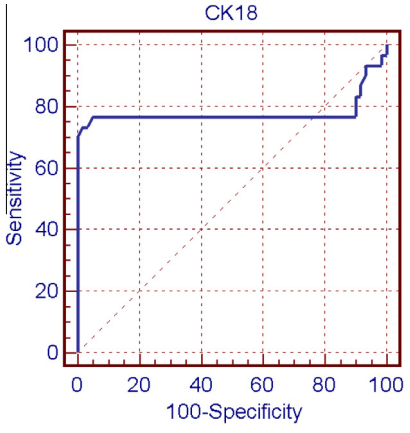

Figure 1 Diagnostic performance of CK18 in diagnosis of NASH. CK18 is highly specific (95%) and moderately sensitive (76.7%) in the diagnosis of NASH.

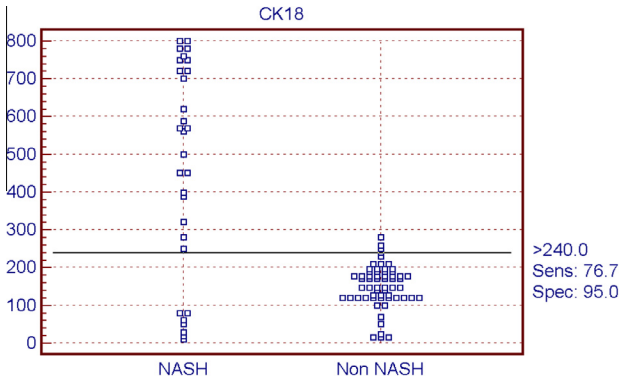

Figure 2 Logistic Regression Test showing positive prediction of CK18 in diagnosis of NASH.

Group I which included 30 patients with NASH. This group included 24 (80 %) males and 6 (20%) females. Their ages ranged from 20 to 74 with mean of 49.86 ± 14.15 years. Their BMI ranged from 35 to 55 with mean of 42.38 ± 4.33. 23/30 of this group was diabetic; 15/30 was hypertensive and 14/30 had dyslipidemia.

Group II which included 30 patients with NAFL. This group included 20 (66.67 %) males and 10 (33.33%) females. Their ages ranged from 19 to 72 with mean of 47.69 ± 11.70 years. Their BMI ranged from 31 to 51 with mean of 41.19 ± 4.24. 20/30 of this group was diabetics; 11/30 was hypertensive and 7/30 had dyslipidemia.

Group III included 30 healthy controls: 22 (73.33 %) males and 8 (26.67%) females. Their ages ranged from 21 to 80 with mean of 47.69 ± 11.70 years. Their BMI ranged from 34 to 49 with mean of 42.96 ± 4.05. 22/30 of this group was diabetic; 8/30 was hypertensive and 6/30 had dyslipidemia.

There is an urgent need to develop and validate a simple, noninvasive test that accurately distinguishes NASH from NAFLD and determines the stage of the disease [12].

In our study we evaluated the role of CK18 in diagnosing NASH and its correlation with disease severity in a sample of Egyptian patients.

In the current study we found statistically significant differences between the 3 groups regarding plasma CK18 levels, being highest in the NASH group (with mean ± SD: 460 ± 279 U/L) when compared with the group of NAFL (mean ± SD: 167 ± 56 U/L) and the control group (mean ± SD: 149 ± 57 U/L), with *P* value <0.001. On the other hand there was no statistically significant difference between the NAFL group and the control group regarding the plasma CK18 level with *P* value >0.05. Our results are similar to Wieckowska et al., who found that CK18 could

Table 1 Comparison between the 3 groups regarding the different parameters.

	NASH no = 30	NAFLD no = 30	Controls no = 30	*P* value	Post hoc test
	Mean ± SD	Mean ± SD	Mean ± SD		
FBS	120 ± 31	76 ± 10	80 ± 10	0.001	**P1 = 0.001***
					P2 = 0.126
					P3 = 0.001*
HBA1C	6.9 ± 1.5	5.0 ± 0.5	5.0 ± 0.5	0.001	**P1 = 0.001***
					P2 = 1.000
					P3 = 0.001*
Chol	232 ± 39	164 ± 31	176 ± 29	0.001	**P1 = 0.001***
					P2 = 0.342
					P3 = 0.001*
TAG	140 ± 61	139 ± 43	144 ± 54	0.933	P1 = 0.157
					P2 = 0.723
					P3 = 0.311
LDL	170 ± 32	82 ± 16	81 ± 13	0.001	**P1 = 0.001***
					P2 = 1.000
					P3 = 0.001*
AST	31 ± 19	28 ± 10	27 ± 10	0.211	P1 = 0.933
					P2 = 1.000
					P3 = 0.811
ALT	55 ± 30	53 ± 18	53 ± 16	0.771	P1 = 0.872
					P2 = 0.951
					P3 = 0.713
Ferritin	260 ± 104	294 ± 113	269 ± 118	0.523	P1 = 0.561
					P2 = 0.813
					P3 = 0.722
CK18	460 ± 279	167 ± 56	149 ± 57	0.001	**P1 = 0.001***
					P2 = 0.872
					P3 = 0.001*

P1 = showing difference between group I (NASH) and group II (NAFLD).
P2 = showing difference between group II (NAFLD) and group III (controls).
P3 = showing difference between group I (NASH) and group III (controls).
Chol: cholesterol, TAG: triglycerides, LDL: low density lipoprotein, CK18: cytokeratin 18.
This table shows significant difference between the NASH and NAFL groups and the NASH and control groups regarding FBS, HA1C, total cholesterol, LDL and CK-18.

Table 2 Correlation between CK-18 and NAS score in group I and II.

CK-18		
	r	*P*-value
Steatosis	0.441	0.015*
Lobular inflammation	0.457	0.011
Cell ballooning	0.509	0.004*
Fibrosis	0.317	0.045*
Mallory bodies	0.470	0.009*
NAS	0.746	0.001*

NAS: NAFLD activity score.
This table shows statistically significant correlation between CK-18 and degree of steatosis, cell ballooning, fibrosis, Mallory bodies and NAS.

Table 3 Correlation between CK18 and other parameters in all groups.

Groups	CK-18	
	r	*P*-value
Sex	0.213	0.699
BMI (body mass index)	0.799	**0.001***
DM (diabetes mellitus)	0.506	**0.0451***
Fasting blood glucose	0.792	**0.001***
Glycosylated hemoglobin	0.810	**0.001***
Hypertension	0.274	0.758
Dyslipidemia	0.458	**0.049***
TAG	0.239	0.933
Cholesterol	0.823	**0.001***
LDL	0.840	**0.001***
AST	0.846	**0.001***
ALT	0.804	**0.001***
Ferritin	0.871	**0.001***

This table shows statistically significant correlation between CK-18 and BMI, DM, FBS, HA1C, dyslipidemia, total cholesterol, LDL, AST, ALT and serum ferritin.

serve as an indicator of hepatic inflammation [13]. Similar results were found in a study done by Feldstein et al. in children, who found that CK18 levels were significantly higher in subjects with NASH compared with NAFL (322.1 U/L ± 104.8 vs. 164.2 U/L ± 62, respectively; $P < 0.001$). They also found that for every 10 U/L increase in CK18 levels, the likelihood of having NASH increased by 70% [14].

This could be explained by the hepatocyte apoptosis process which is one of the key components involved in the progression of NAFL to NASH. This process is mediated by the caspase activity. It is reported that Caspase 3 is activated in NASH liver, which induce hepatocyte apoptosis and release the CK18 fragments into the sera of NASH patients [15].

In a study done by Gonsebatt et al., hepatic mRNA expression of CK18 has been reported to be up-regulated by oxidative stress in mice; which could be another explanation for the elevated levels of serum CK18 in NASH patients [16].

In the current study, there was positive statistically significant correlation between CK18 and BMI, DM, fasting blood sugar, glycosylated hemoglobin (HBA1C), dyslipidemia, cholesterol level, LDL, AST, ALT, ferritin and NAS. These results go with the results of Miyasato et al. who found significant association between CK18 and BMI in the T2DM patients with NAFLD [17].

In another study, CK-18 correlated most strongly with ALT and adipose tissue insulin resistance (IR), less with steatosis, lobular inflammation and fibrosis, but not with ballooning, BMI, metabolic syndrome or T2DM [18].

In the current study, there was positive correlation between CK-18 and NAS score ballooning, Mallory bodies formation, steatosis and fibrosis in both the NASH and simple steatosis groups.

Diab et al., showed a significant positive correlation between serum levels of fragmented CK18 and NAS in 65 NAFLD patients who had undergone bariatric surgery, and a dramatic decrease in CK-18 levels 6 months following surgery. These changes were greater in those subjects with NASH and positively correlated with changes in transaminases, suggesting that measuring CK-18 fragment levels in the blood may be a useful test to monitor disease status postoperatively [19].

The results of the current study goes with the results of the study of Vuppalanchi et al., who found that changes in serum levels of cytokeratin fragments appear to reflect changes in liver histology in patients with NASH. They found that every 100-U/L decline in serum cytokeratin fragment (CK-18) level was significantly associated with overall histological improvement ($P < 0.001$); resolution of NASH ($P = 0.002$); and improvement of at least 1 point in steatosis grade, hepatocellular ballooning, and nonalcoholic fatty liver disease (NAFLD) score (P less than .001 for all) [20].

In the current study, the ROC curve shows diagnostic performance of CK18 with specificity (95%) and sensitivity (76.7%) in the diagnosis of NASH with serum levels >240 U/L. The Logistic Regression Test shows positive prediction of CK18 in diagnosis of NASH.

Although Younossi et al. proved the independency of CK18 fragments as a predictor of NASH, their results suggest that CK18 fragments have sufficient-to-excellent diagnostic accuracy for the detection (or exclusion) of NASH in the setting of NAFLD [21]. As a consequence, Younossi and coworkers have developed a test (NASH diagnostics) based on four ELISA assays (cleaved and intact CK18 added to serum adiponectin, and serum resistin) to form a simple diagnostic marker for NASH [21].

Table 4 ROC curve showing diagnostic performance of CK18 in diagnosis of NASH.

ROC curve					
Cutoff	Sensitivity (%)	Specificity (%)	PPV	NPV	Accuracy
>240 U/L	76.7	95.0	88.5	89.1	87.12

PPV: positive predictive value.
NPV: negative predictive value.

In another study done by Feldstein et al., the performance of CK18 level for the diagnosis of NASH was excellent with an area under the Receiver Operating Characteristics curve of 0.933 [14] (Figs. 1 and 2 and Tables 1–4).

5. Conclusion

Measurement of CK18 seems to be promising as screening, diagnostic and staging biomarker in NASH.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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