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ORIGINAL ARTICLES

CYTOKERATIN-18 AS A NONINVASIVE BIOMARKER OF INFLAMMATION IN NONALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

INTRODUCTION: Nonalcoholic fatty liver disease (NAFLD) is a public health problem of global significance. It is defined as the presence of hepatic steatosis in more than 5% of hepatocytes, as determined by imaging or histological examination, in individuals consuming little or no alcohol, and in whom a secondary cause of steatosis has been excluded. Because of the proven association between NAFLD and metabolic syndrome (MetSyn), an international panel of experts has proposed to replace the term non-alcoholic fatty liver disease with the term metabolic-associated fatty liver disease (MAFLD). Nonalcoholic fatty liver disease is divided into two main groups: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). Multiple serum biomarkers have been investigated to predict the presence of NASH. Cytokeratin-18 (CK18) is the only validated biomarker. It is a marker of hepatocyte apoptosis and predicts presence of steatohepatitis.

AIM: The aim of this article is to evaluate the application of CK18 as a diagnostic marker of inflammation in NAFLD and its relevance using anthropometric parameters, routine laboratory tests, ultrasonographically defined degree of steatosis, and stage of fibrosis calculated from non-invasive scores.

MATERIALS AND METHODS: The study included 61 persons with ultrasonographycally proven NAFLD (mean age 56.9 years). Data on anthropometry, clinical features, standard laboratory tests, ultrasound examination, and serum levels of total CK18, determined by the ELISA method, were collected. Using some laboratory parameters and clinical characteristics of the patients, scores for non-invasive assessment of fibrosis were calculated.

RESULTS: Normal CK18 levels were found in 55 patients (90.17%), and an increase in CK18 above 5 ng/mL, indicating the presence of steatohepatitis, was found in 6 patients (9.83%), of which 2 males and 4 females. In CK18 levels above 5 ng/mL (ULN), we accepted a diagnosis of steatohepatitis. We found moderately strong positive correlation between the level of triglycerides and CK18, indicating an increase in the levels of triglycerides in parallel with the CK18 levels. Such correlation was not found with the increase of body mass index (BMI) and waist circumference and the increase in CK18 levels. Significantly increased AST, ALT, and GGT levels were observed in the group of patients with CK18 above 5 ng/mL, compared to the patients in the group with normal CK18. In our study, the correlation analysis between the level of CK18 and the degree of steatosis and the stage of fibrosis did not find a statistically significant correlation.

CONCLUSION: We assumed that the detection of elevated CK18 levels is a reliable method to prove inflammation in NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a public health problem of global significance, affecting approximately one million individuals worldwide. It is defined as the presence of hepatic steatosis in more than 5% of hepatocytes, as determined by imaging or histological examination, in individuals consuming little or no alcohol, and in whom a secondary cause of steatosis has been excluded. Nonalcoholic fatty liver disease is divided into two main groups: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). Nonalcoholic fatty liver is considered to be the non-progressive form of NAFLD, with minimal or no progression to cirrhosis and no liver-related mortality, while NASH is the progressive form, leading to the development of fibrosis, cirrhosis, hepatocellular carcinoma, and increased liver-related mortality. The active form of NASH is characterized histologically by the presence of inflammation and hepatocyte ballooning degeneration, which determines the faster progression of the disease (1–4).

Nonalcoholic fatty liver disease arises from the interaction of environmental, epigenetic, and genetic factors and is directly related to the presence of insulin resistance. It is associated with the presence of obesity, type 2 diabetes mellitus (DM), hypertension, and metabolic syndrome (MetSyn) (5). Because of the proven association between NAFLD and MetSyn, an international panel of experts has proposed to replace the term *non-alcoholic fatty liver disease* with the term *metabolic-associated fatty liver disease* (MAFLD) (6). Due to the global obesity epidemic, NAFLD has emerged as a leading cause of liver-related morbidity and is predicted to become a major cause of liver transplantation in the next decade.

A large proportion of patients with NAFLD do not experience disease progression, only patients with NASH and advanced fibrosis are at risk of developing advanced liver disease with complications (7). Therefore, distinguishing NASH from pure hepatic steatosis is of great importance in patients with NAFLD. Still, the gold standard for establishing the diagnosis of steatohepatitis is histological examination. Liver biopsy has its well-known limitations. As a result of the disadvantages of liver biopsy, various alternative non-invasive biomarkers to identify patients with NASH and advanced fibrosis from the general group of NAFLD patients have been intensively investigated and used over the past decade. There is a lack of non-invasive tests, especially for detecting the presence of inflammation. All non-invasive methods for NAFLD staging are still under investigation and research, and a universally accepted international standard is lacking.

Multiple serum biomarkers have been investigated to predict the presence of NASH. Cytokeratin-18 (CK18) is the only validated biomarker, in over 10 studies with more than 1000 patients (8,9). Fragments of CK18 are markers of hepatocyte apoptosis (M30 fragment) and are formed during cell death (M65 fragment) as a result of the action of the enzyme caspase 3. It is measured by an immunoenzymatic method. M30 ELISA measures cleaved caspase fragment and demonstrates apoptosis, which is characteristic of steatohepatitis, and M65 ELISA is a reflection of total cell death.

Apoptosis is a physiological process of programmed cell death occurring at the cellular level. Its aim is more effective adaptation of the organism to its surroundings. Unlike necrosis, which is a pathological process and is the result of unfavorable external influences, apoptosis begins intracellularly. It is associated with numerous biochemical processes causing irreversible morphological changes—DNA fragmentation, changes in the cell membrane, chromatin condensation and others, incompatible with the normal functioning and survival of the cell.

As a result of cell death, components of the cellular structure, reflecting the apoptosis that has occurred, enter the plasma. Such are cytokeratins, representatives of the intermediate filaments of epithelial cells. Cytokeratin-18 is a specific intermediate filament of the epithelial cells—liver, sinusoidal, bile duct cells, but not of mesenchymal cells. It represents about 5% of the total protein in the liver, exocrine pancreas, small intestine, and other tissues. It is a structural component of the nuclear lamina (10,11).

The first large study by Feldstein and co-authors in 2009 (12) found that circulating serum levels of CK18 above 250 U/mL could be predictive of the presence of steatohepatitis among patients with NAFLD. Cytokeratin-18 levels were much higher in patients with steatohepatitis than in those with sim-

ple non-progressive steatosis. Many other studies subsequently confirmed these results, but in a smaller population. A meta-analysis showed an AUROC of 0.82 with a sensitivity of 78% and a specificity of 86% (13). The total level of CK18 showed similar accuracy in establishing NASH. A pilot Bulgarian study by Marinova, presented in a dissertation, examined the level of CK18 in 65 patients, of which 20 had NAFLD; the rest had chronic hepatitis C (CHC), chronic hepatitis B (HCV), and 20 healthy controls (14).

Cytokeratin-18 is the most promising standalone parameter for distinguishing steatosis from NASH. As a single marker of apoptosis/necrosis and an indicator of liver inflammation, it is suggested that CK18 may be applied to differentiate simple non-progressive steatosis from steatohepatitis, especially in cases with NAFLD, and is useful to predict more active and progressive disease and define the risk groups of patients in whom active treatment should be initiated. Experience with the use of CK18 in routine practice is lacking, and there is variability in the probable cut-off values and their respective diagnostic accuracy among different studies. There is no large study among the Bulgarian population focusing on the relationship between CK18 and NAFLD.

AIM

The aim of this article is to analyze the application of CK18 as a diagnostic marker of inflammation in NAFLD and its relevance with anthropometric parameters, routine laboratory tests, ultrasonographically defined degree of steatosis, and stage of fibrosis calculated from non-invasive scores.

MATERIALS AND METHODS

A total of 61 individuals were included in the study. They were examined at the Gastroenterology Clinic at St. Marina University Hospital in Varna for the period from June 2021 to May 2022. All patients were with ultrasonographically proven NAFLD.

Exclusion criteria were the presence of alcoholic liver disease, with anamnestic evidence of absolute alcohol consumption >20 g per day for women and >30 g per day for men, evidence of secondary non-alcoholic steatosis (chronic hepatitis C or B, Wilson's disease, autoimmune liver disease, intake of steatogenic drugs, etc.), presence of functional class III-IV heart failure according to NYHA, as well as other accompanying diseases that would affect the obtained results. The study was approved by the Research Ethics Committee at MU-Varna with protocol No. 76/09.08.2018. All patients signed an informed consent form to participate in the clinical observation.

In all patients complete clinical examination, including measurement of anthropometric parameters—height (cm), body mass (kg) and waist circumference, laboratory and ultrasound examination, were performed. Standard laboratory tests were conducted in the morning on an empty stomach (no food intake for at least 8 hours before the test). They included: hematological parameters—hemoglobin, hematocrit, erythrocytes, leukocytes, platelets; standard biochemical parameters, incl. blood glucose, HbA1C, AST, ALT, GGT, AF, total and direct bilirubin, cholinesterase, total protein, albumin, CRP, serum iron, ferritin, lipids—total cholesterol, HDL, LDL, TG, coagulation status—PI%.

Serum CK18 was studied in all 61 subjects with evidence of hepatic steatosis, of whom 23 were men and 38 were women. The mean age of the study group was 56.19 years, with a minimum age of 28 years and a maximum age of 79 years. We quantitatively measured the levels of total serum CK18 by the sandwich ELISA method, which uses specific antibodies—anti-human CK18 (Millipore). The tests were carried out in the Central Clinical Laboratory of St. Marina University Hospital. The measurement units of the obtained results were in ng/mL. According to the kit manufacturer's instructions, a reference level of 5 ng/mL was taken. The measured mean level was 3.01±10.42 ng/mL, with a minimum level of 0.1 ng/mL and a maximum level of >80 ng/mL.

All patients underwent a standard ultrasound examination of the abdominal organs with an Aloka Prosound Alpha 7 ultrasound machine with a convex transducer with a frequency of 2.2–2.5 MHz. A complete examination of abdominal organs was carried out—liver, gall bladder, pancreas, spleen, kidneys, small pelvis, and bowels.

Using specific laboratory parameters and clinical characteristics of the patients, the following scores were calculated to assess fibrosis: APRI score (AST to platelet ratio index (APRI)), fibrosis-4 (FIB-

4), NFS (NAFLD fibrosis score), BARD score, and AST/ALT ratio.

The present work included various descriptive and analytical methods based on parametric and non-parametric tests addressing the research objectives. Descriptive methods, analytical methods, correlation analysis, and regression analysis were used.

RESULTS

The characteristics of the studied group with CK18 showed an average value of BMI—34.73 ± 7.23, with a minimum value of 24.8 and a maximum value of 66.98. The average waist circumference was 115.19±12.83 cm, 119.34±13.01 cm for men, and 112.68±12.21 cm for women (Table 1). Of all 61 patients examined, 47 (77.04%) had evidence of registered hypertensive heart disease (HD), and 14 did not have elevated blood pressure. In 20 patients (32.78%), type 2 DM was established, respectively 41 patients (67.22%) had no data for DM. In 8 (13.11%) of the patients, all 3 acceptance criteria for the presence of metabolic syndrome were missing, and 1 or 2 risk factors were present.

Laboratory tests in the group showed an increase in ALT above normal in 11 patients, with a maximum level of 146 U/L and a minimum level of 5 U/L, mean level 32±32.09 U/L. The average level of AST was 28.78±19.94 U/L, with a maximum of 114 U/L and a minimum of 13.4 U/L. We found AST increase above ULN in 6 patients. Gamma-glutamyl transferase (GGT) was within ULN and different in both sexes. The mean level in men was 39.69±22.14 U/L, with a minimum of 12 U/L and maximum of 122 U/L. We observed an increase in only 1 patient. In women, the mean GGT level was 44.39±43.98 U/L, with a minimum of 8 U/L and a maximum 178 U/L, and an increase was observed in 13 patients. The mean level of triglycerides was 2.13±1.01 mmol/L, with a minimum of 1.07 mmol/L and a maximum of 4.92 mmol/L, and an increase above ULN was observed in 13 patients. Albumin and platelet levels were within reference limits in all patients.

All patients (n=61) had sonographic evidence of steatosis, mild in 11 patients, moderate in 23, and severe in 27. The degree of fibrosis was determined based on the calculated scores. The determined FIB-4 in the study group showed a mean value of 1.25±0.69, a minimum of 0.33 and a maximum of 3.33. There were 36 patients with adherent or mild fibrosis with a score below 1.3, 22 patients with a moderate fibrosis score between 1.3 and 2.67, and 3 patients with advanced fibrosis above 2.67. The NAFLD fibrosis score had a mean value of -1.27±1.44, a minimum of -3.81 and a maximum of 2.58. In the studied group with absent or mild fibrosis with a score below -1.45, there were 26 patients (42.7%), in the one with a moderate fibrosis score between -1.45 and 0.675, there were 29 patients (47.5%), and in that with advanced fibrosis, there were 6 patients (9.8%).

Among the studied group, normal CK18 levels were found in 55 patients (90.17%), and an increase in CK18 above 5 ng/mL, indicating the presence of steatohepatitis, was found in 6 patients (9.83%), of which 2 males and 4 females (Fig. 1). In CK18 levels above 5 ng/mL (ULN), we accepted a diagnosis of steatohepatitis.

Table 1. Characteristics of patients tested for cytokeratin-18 in terms of age, BMI and abdominal circumference.

	Age	BMI	Waist Circumference
Average value	56.19	34.73	115.19 cm
Standard deviation	± 3.01	± 7.23	± 12.83 cm
Minimum value	28	24.8	96 cm
Maximum value	79	66.98	146 cm

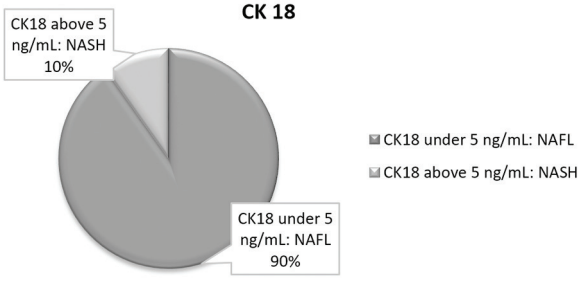

Fig. 1. Distribution by elevated CK18 levels—NASH/NAFL.

Correlation analysis according to the Pearson method was used to study the relationship between the clinical parameters and establish the strength of their influence on cytokeratin levels. The results of the correlation analysis showed the presence of a moderately strong positive correlation between the level of triglycerides and CK18 (r=0.318, p=0.012), indicating an increase in the levels of triglycerides in parallel with the CK18 levels. Such a correlation was not found regarding the increase in BMI and waist circumference on the one hand and the increase in CK18 on the other.

The investigated correlation by the Spearman method between the presence of HD, DM, and MetSyn with CK18 levels did not produce significant results. Type 2 DM 2 was compared to CK18 levels (rho=0.082, p=0.532), HD was compared to CK18 levels (rho=0.176, p=0.174), and MetSyn was compared to CK18 (rho=-0.107, p=0.414).

Correlation analysis according to the Pearson method for the AST, ALT, GGT, CRP laboratory parameters and CK18 did not show significant correlation. We considered this lack of correlation to be due to the lack of large variation in Ck18 levels in our study group of patients (n=61). In order to avoid this circumstance with the help of independent samples t-test, we divided the patients with tested CK18 into two groups—those with a normal CK18 levels and a group with CK18 levels above 5 ng/ml. The purpose of the analysis was to compare the average measured levels of AST, ALT, GGT, and CRP between the two groups (Table 2).

The results showed that for AST the mean level was 25.9±16.4 U/L for the patients of the first group with normal CK18, and for the second group with CK18 above 5 ng/mL, the mean level was 54.93 ± 31.06 U/L. Therefore, significantly increased AST levels were observed in the second group, and these differences were statistically significant (t=-3.729, p=0.001). Regarding ALT, the mean level was 28.2±19.10 U/L for the patients of the first group with normal CK18, and in the second group with CK18 above 5 ng/mL, the mean level was significantly higher: 66.63±55,65 U/L. Therefore, significantly increased ALT levels were observed in the second group, and these differences were statistically significant (t=-2.958, p=0.004). The average GGT level in the first group was 39.2±34.3, and in the group with elevated CK18, it was 74.17±49.97, the difference being statistically significant (t=-2.266, p=0.027). Only with regard to CRP levels, there were no statistically significant differences between the two groups (t=0.585, p=0.563) (Fig. 2).

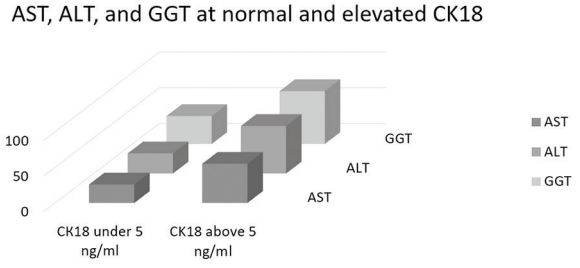

Fig. 2. Comparison of the average AST, ALT, and GGT levels in groups with normal and elevated CK1.8

In order to investigate whether there is a correlation between the CK18 level and the degree of steatosis determined sonographically, we performed a correlation analysis, which did not show a statistical significance (rho=0.147, p=0.259).

We performed a correlation analysis between the CK18 level and the fibrosis scores (FIB-4 index, APRI, and NAFLD fibrosis score), but we did not establish a statistically significant correlation. With the help of independent samples t-test, we again divided the patients with tested CK18 into two groups—

Table 2. Independent samples t-test comparing the mean levels of AST, ALT, GGT, and CRP between normal and elevated CK18 groups.

Independent Samples T-Test					
		t		df	p
AST	Student's t	-3.729	[a]	59.0	<.001
ALT	Student's t	-2.958	[a]	59.0	0.004
GGT	Student's t	-2.266		59.0	0.027
CRP	Student's t	0.585		30.0	0.563

[a] Levene's test is significant (p < .05), suggesting a violation of the assumption of equal variances

those with a normal CK18 levels and a group with CK18 above 5 ng/mL, and compared the mean levels of FIB-4, APRI, and NAFLD fibrosis score between the two groups. The results showed no statistically significant difference between the two groups for the NAFLD fibrosis score (t=0.151, p=0.880) and FIB-4 (t=-1.936, p=0.058). Only for APRI there was a statistically significant difference established (t=-3.872, p=0.001).

These data are explained by the fact that, in general, in the studied group of patients, those with advanced fibrosis, determined according to the value of the fibrosis scores, were a very small proportion. The majority of patients either had no fibrosis or fell into the gray area with mild fibrosis. Regarding the APRI score, the patients were also without significant fibrosis, but this score was calculated on the basis of only two parameters. One of them was AST and, as we found out, in patients with elevated CK18, there was also a statistically significant increase in transaminases, which explains the described correlation with APRI.

DISCUSSION

In our study the correlation analysis used to examine the correlation between clinical parameters and establish the strength of their influence on CK18 levels showed a moderately strong positive correlation between the level of triglycerides and CK18, indicating an increase in the levels of triglycerides in parallel with the CK18 levels. The correlation between the increase of triglycerides and the inflammatory status determined by the CK18 level has led us to the conclusion that the increased level of triglycerides is associated with a high probability of inflammatory processes. No correlation was found regarding the increase in BMI and abdominal circumference on the one hand and the increase in CK18 on the other. Correlation analysis did not give significant results for the correlation between the increase in the CK18 level and the presence of HD, DM, and MetSyn.

Our study showed significantly increased ALT, AST, and GGT levels in the group with elevated CK18 compared to that with normal, and the differences found were statistically significant. The abovementioned results give us reason to assume that the detection of elevated CK18 is a reliable method to prove inflammation in NAFLD. Statistically significant associations were not found between CRP and CK18 levels.

According to literature data, an increase in CK18 correlates with a higher degree of fibrosis. In our study, the correlation analysis between the level of CK18 and the degree of steatosis and the stage of fibrosis did not produce a statistically significant result. These data are explained by the fact that in the studied group few patients had advanced fibrosis, determined according to the value of the fibrosis scores. Patients with absent or mild fibrosis predominated. A larger sample of patients with different liver densities is likely needed to conclude whether elevated CK18 levels correlate with higher-grade fibrosis. We conclude that CK18 is a reliable marker of hepatocyte inflammation in routine practice.

CONCLUSION

The spreading global epidemic of obesity is leading to a parallel increase in the incidence of NAFLD, making it a leading cause of liver damage. It is predicted that NAFLD will soon overtake other liver diseases in terms of morbidity, mortality, and become the leading cause of liver transplantation. All these facts emphasize the importance of making an adequate diagnosis and staging of NAFLD. Therefore, there is a need for easily accessible, highly sensitive and specific tests that allow not only the identification of patients at high risk of aggressive disease outcome, but also enable the monitoring of disease progression and therapeutic response. Detection of elevated serum CK18 as a single non-invasive marker of hepatocyte necrosis/apoptosis is a reliable method to determine inflammation in NAFLD.

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