Diagnostic and prognostic potential of kallistatin in assessment of liver parenchyma changes in patients with non-alcoholic fatty liver disease and hypertension

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ABSTRACT

Background and aim: Non-alcoholic fatty liver disease (NAFLD) is closely linked to hypertension (HT). An important issue remains the search for non-invasive tests to NAFLD detection in the early stages of liver fibrosis. The objective of the study was to evaluate the diagnostic and prognostic value of kallistatin in assessing the liver fibrosis progression in NAFLD and HT patients.

Patients and methods: One hundred fifteen patients with NAFLD with and without HT were examined, the control group consisted of 20 relatively healthy volunteers. Plasma kallistatin level measurement, ultrasound steatometry and elastography were performed in all patients.

Results: Kallistatin level was 65.03 ng mL⁻¹ (95% CI 61.38; 68.68), 83.42 ng mL⁻¹ (95% CI 81.89; 84.94) and 111.70 ng mL⁻¹ (95% CI 106.14; 113.22) in patients with NAFLD and HT, isolated NAFLD and control group, respectively. There were significant differences in the liver parenchyma condition between groups. Kallistatin levels strongly inversely correlated with the attenuation coefficient and the mean liver stiffness in NAFLD and HT (rs = −0.70) and in the isolated NAFLD patients (rs = −0.56; rs = −0.68, respectively). Kallistatin level was 71.82 ng mL⁻¹ (95% CI 70.16; 79.51) and 58.62 ng mL⁻¹ (95% CI 55.81; 64.45) in patients with HT stage I and HT stage II, respectively (P < 0.001).

Conclusions: Concomitant HT in NAFLD patients is associated with greater severity of fatty and fibrotic liver changes. The course of NAFLD is accompanied by decrease in kallistatin level. Increased degree of liver steatosis and fibrosis, inflammation activity, increased BMI and increased stage of HT lead to inhibition of kallistatin activity. Kallistatin may be considered as a biomarker for progression assessment of NAFLD with or without HT.

KEYWORDS

kallistatin, non-alcoholic fatty liver disease, NAFLD, hypertension, ultrasound steatometry, ultrasound elastography

Introduction

Non-alcoholic fatty liver disease (NAFLD) is rightly considered to be the one of the most common chronic liver diseases. The prevalence of pathology in the world averages 25.2% with wide geographic variations depending on the level of medical diagnostic capabilities in the region [1]. The prevalence of non-alcoholic steatohepatitis (NASH), as a more severe type
of NAFLD, is estimated at 1.5–6.5% in the general population, and approximately 41% of patients with NASH have progression of liver fibrosis [2, 3]. Considerable attention is paid to the close relationship between NAFLD and cardiovascular diseases (CVD), especially to comorbidity with arterial hypertension (HT) that affects about 30% of the world’s adult population [4]. The prevalence of HT is significantly higher in individuals with NAFLD than in the general population, and up to 49.5% of patients with hypertension have NAFLD. In turn, patients with fatty liver have a three times higher risk of hypertension [5].

Through common pathogenetic links, NAFLD can both precede and contribute to the development of hypertension, and occur against the background of HT, regardless of other metabolic disorders [6]. It is also known that CVD is the most common cause of death in NAFLD patients [7]. Therefore, early diagnosis of liver fibrotic changes is important for the prevention of NASH, especially in the case of a comorbid combination of NAFLD and HT.

The “gold standard” for the diagnosis of NAFLD is liver biopsy. However, this expensive invasive procedure is associated with a high risk of complications, frequent refusal of examination by patients, as well as 10–30% of false negative results in patients with NASH [8, 9]. In recent decades, it is considered expedient to study the level of serum and plasma biomarkers to create models for steatosis and fibrosis diagnosing and predicting the course of NAFLD [10, 11].

Kallistatin (kallikrein-binding protein) is a recently identified member of the serine protease inhibitor family that synthesized and expressed mainly in the liver and distributed between the tissues of the heart, kidneys and blood vessels [12]. This protein maintains blood pressure (BP) levels and exhibits anti-inflammatory, antioxidant, antiangiogenesis, and antitumor effects [13–16]. Various studies confirm that kallistatin can be an effective biomarker for the early detection of liver fibrosis in different liver diseases [17–19].

However, the potential anti-inflammatory and biomarker role of kallistatin in the NAFLD course requires further study.

The Objective of the Study was to evaluate the diagnostic and prognostic value of kallistatin in assessing the liver fibrosis progression in patients with NASH in combination with HT.

Patients and methods

The study was conducted at the Department of Internal Medicine No. 1 of Kharkiv National Medical University from September 1, 2018 to January 31, 2020. The study was approved by the Ethics and Bioethics Commission of Kharkiv National Medical University. The experimental part of this study respects the ethical standards in the Helsinki Declaration of 1975, as well as the principles of Good Clinical Practice (GCP) and the national law. All patients voluntarily decided to participate in the study and signed the patient’s informed consent.

The inclusion criteria were the presence of NAFLD with or without concomitant HT, age 18–60 years. The exclusion criteria were: other diffuse and focal liver diseases (alcoholic fatty liver disease, viral hepatitis, liver cirrhosis, etc.); HT III stage, 3 grade; coronary heart disease; diabetes mellitus and other endocrine pathologies; oncological diseases; systemic connective tissue diseases; the presence of symptomatic arterial hypertension; acute and chronic internal inflammatory diseases; patient’s refusal to give informed consent.

We examined 115 patients with NAFLD at the stage of non-alcoholic steatohepatitis (NASH). Participants included 57 men and 58 women aged 38–59 years (M = 48.4; 95% CI 47.4; 49.3). The patients were divided into two groups depending on the presence of concomitant HT. The main group (n = 63) consisted of 32 men and 31 women aged 38–59 years (M = 48.4; 95% CI 47.2; 49.6) with NAFLD and HT, and the comparison group (n = 52) included 25 men and 27 women aged 39–59 years (M = 48.3; 95% CI 46.8; 49.8) with an isolated course of NAFLD. The groups had no differences in age (P = 0.908), gender composition (df = 1, \( \chi^2 = 0.084, P = 0.772 \)). The control group (n = 20) consisted of practically healthy 8 men and 12 women aged 38–56 years (M = 47.1; 95% CI 45.1; 49.1), randomized by age (\( p = 0.394; P = 0.555 \)) and sex (\( df = 1, \chi^2 = 0.380, P = 0.400; df = 1, \chi^2 = 0.708, P = 0.538 \)).

In the main group of patients the NAFLD duration was on average 6.6 years (95% CI 5.81; 7.32), and the duration of HT averaged 8.4 years (95% CI 7, 34; 9.48). At the same time, in the group of patients with isolated NAFLD, the duration of the underlying disease averaged 7.8 years (95% CI 6.70; 8.84), and there was no significant difference in the duration of NAFLD between two groups of examined patients (P = 0.086).

NASH was diagnosed in the previous stages of the study based of laboratory and instrumental examinations in accordance with EASL-EASD-EASO clinical practice guidelines (2016) [20]. Given the absence of severe liver fibrosis and cirrhosis, the combination of transient elastography and serum markers avoided the need to perform a liver biopsy in these patients.

The diagnosis of HT was also confirmed in the previous stages of the study according to the ESH/ESC Clinical Practice Guidelines for the Management of Arterial Hypertension (2018) [21].

During an objective examination, body mass index (BMI) was calculated for all patients by conventional formula: BMI = body weight (kg)/height (m2), and the value of office systolic (SBP) and diastolic (DBP) blood pressure was determined in accordance with the ESH/ESC Clinical Practice Guidelines (2018) [21].

Biochemical parameters of liver functional activity were determined by spectrophotometric methods (including alanine aminotransferase (ALT) and aspartate aminotransferase (AST)).

Plasma kallistatin levels were determined using the enzyme-linked immunosorbent assay using the Human SERPINA4 (Kallistatin) ELISA Kit (Elabscience, USA).
level of C-reactive protein (CRP) was determined using the hs-CRP ELISA Kit (Biomerica USA).

Based on the obtained clinical and laboratory data, the Non-alcoholic Fatty Liver Disease Fibrosis Score (NFS) index was calculated by the formula: $-1.675 + (0.307 \times \text{age (years)}) + (0.094 \times \text{BMI (kg m}^{-2}) + (1.13 \times \text{violation glucose tolerance/diabetes mellitus (yes = 1, no = 0)}) + (0.99 \times \text{AST/ALT}) - (0.013 \times \text{platelet level ($\times 10^{12}$/L)} - (0.66 \times \text{albumin (g dL}^{-1}))$, with higher marker scores, a higher risk of fibrosis was revealed [11].

The parameters of hepatic steatosis and fibrosis were assessed using a Sonus P7 ultrasound system (Ultrason, Ukraine). In the “AC” mode, a quantification of hepatic steatosis was carried out using measuring the value of the liver parenchyma linear attenuation coefficient (dB cm$^{-1}$). The liver fat infiltration was assessed according to ultrasound attenuation scale (Sasso M. et al. [22], the validated scale (NAS – NAFLD activity score) and the Sonus P7 correspondence table. The Attenuation coefficient (AC) in the range 1.0–2.19 dB cm$^{-1}$ corresponded to the S0 stage according to NAS (no steatosis, hepatocytes with macrovesicular steatosis – from 0 to 5%, 2.2–2.29 dB cm$^{-1}$ – S1 (mild steatosis, hepatocytes with macrovesicular steatosis – >5–33%), 2.3–2.9 dB cm$^{-1}$ – S2 (moderate steatosis, hepatocytes with macrovesicular steatosis – >33%) and 2.9–3.5 dB cm$^{-1}$ – S3 (severe steatosis, hepatocytes with macrovesicular steatosis – >66%) stages, respectively.

In the “SE” mode, the liver fibrosis degree was determined using shear wave elasticity imaging and elastometry with liver stiffness (kPa or m s$^{-1}$) measuring. 2D shear wave elastography (2D-SWE) was provided by one of three sonographers with an average of 10 years (range 7–16 years) of experience. Before the examination, the patients followed the fasting conditions. The examinations were performed using a convex sensor 2.5–5 MHz at a depth of 10–50 mm from the liver capsule and right intercostal approach, measurements were obtained when patients hold their breath on exhalation. The region of interest (ROI) box size was 2–4 cm laterally and 3–5 cm axially. We obtained five separate 2D-images from similar ROI. Measurements were considered reliable when the elastogram was stable during not less than three seconds before image obtaining and the ROI was color-filled homogeneously.

The measurement results were analyzed using the correspondence of the liver stiffness (kPa; m s$^{-1}$) to the fibrosis and cirrhosis stages according to the METAVIR scale (Castera et al. [23]) and the Sonus P7 correspondence table. Based on these data, we considered cases of liver stiffness (LS Me) in the range of 2.5–6.0 kPa as F0 stage according to METAVIR score (no fibrosis), 6.0–7.0 kPa as F1 (weak fibrosis, star-shaped expansion of portal tracts by fibrosis without septal formation), 7.0–9.5 kPa as F2 (mild fibrosis, portal tracts expansion with single porto-portal septa (>1 septi)), 9.5–12.5 kPa as F3 (severe fibrosis, numerous porto-central septa), 12.5–60 kPa as the F4 (cirrhosis) stages, respectively.

The obtained results were statistically processed using “Excel 2019” (Microsoft) and “STATISTICA 8.0.” (StatSoft Inc.) PC-programs. Continuous variables were presented as mean (M) or median (Me) depending on the sampling distribution and confidence intervals (CI) with a specified reliability $\gamma = 0.95$ (95% CI). The significance of differences between the relative indicators in the groups was confirmed using the Pearson $\chi^2$ test. Statistically significant differences between the traits level in two different groups were determined using the Mann-Whitney U test. The relationship between the two independent indicators was determined using the Spearman’s rank correlation coefficient (rs), the strength of the relationship was assessed according to the Evans scale (J.D. Evans, 1996) [24]. The results of all statistical analysis calculations were considered reliable if $P < 0.05$.

## Results

During the examination, there were determined 12 men and 9 women with HT I (the average age was 43.0 years (95% CI 44.5; 49.3)), 20 men and 22 women with HT II (mean age 49.1 years (95% CI 47.7; 50.5); $P < 0.05$).

The BMI of patients with NASH and HT was 26.9 (95% CI 24.45; 29.34), while in the group with isolated NASH the BMI was 25.1 (95% CI 25.38; 26.56). The BMI of the control group averaged 22.7 (95% CI 22.41; 23.46). The indices in the groups with comorbid and isolated NASH had no significant differences ($P = 0.477$), but in both groups they far exceeded the control BMI ($P < 0.001$).

After measuring BMI, it was revealed that 22 patients with NASH and HT and 26 patients with isolated NASH had normal body weight (BMI = 20–24.9), while 41 patients with NASH and HT and 26 patients with isolated NASH were overweight (BMI = 25–29.9) ($df = 1$, $\chi^2 = 2.664$, $P = 0.103$). SBP levels were 140 mm Hg (95% CI 137.86; 140.55) and 120 mm Hg (95% CI 120.83; 122.24), while DBP was 85 (95% CI 82.72; 86.17) and 70 (95% CI 70.54; 73.30) in the groups with comorbid and isolated NASH, respectively. BP levels significantly differed between groups ($P < 0.001$).

The ALT values were higher both in patients with NASH and HT (79.00 IU/L (95% CI 80.00; 86.98), $P < 0.001$), and in patients with isolated NASH (69.00 IU/L (95% CI 65.29; 70.79), $P < 0.001$) compared with the control values (20.00 IU/L (95% CI 18.77; 23.92)), and also significantly differed among two groups ($P < 0.001$).

AST levels were 75.05 IU/L (95% CI 68.13; 75.17) in the main group, which is higher than in the comparison (54.00 IU/L (95% CI 53.16; 56.99), $P < 0.001$) and control groups (16.5 (95% CI 15.36; 20.04), $P < 0.001$).

CRP levels in NASH and HT patients averaged 7.90 mg L$^{-1}$ (95% CI 7.96; 8.75) and in isolated NASH patients it was 6.55 mg L$^{-1}$ (95% CI 6.47; 7.57) that exceeded the control values (2.07 mg L$^{-1}$ (95% CI 1.83; 2.85), $P < 0.001$).

The results of the NFS index calculations in patients of the main group were $-1.659$ (95% CI $-1.656; -1.158$), which was higher than in comparison ($-2.448$ (95% CI
Ranking hepatic steatosis and fibrosis (Fig. 3) according to the NAS and METAVIR scales showed that the fatty liver infiltration ($P = 0.005$), as well as liver fibrotic changes ($P < 0.001$) increase due to concomitant hypertension in NASH. This is evidenced by a significant decrease in the incidence of mild steatosis and fibrosis – S1 and F0 – and a corresponding increase in S3 and F2 in patients with comorbid pathology (Table 1).

Kallistatin levels strongly inversely correlated with the AC and the LS values in the group of patients with NASH and HT ($rs = -0.70$). In the group of patients with isolated NASH, the level of kallistatin moderately inversely correlated with the AC-Median ($rs = -0.56$), and had strong correlations with the average liver stiffness ($rs = -0.68$). The results obtained indicate an accelerated progression of liver steatosis and fibrosis in the case of reduced anti-inflammatory activity of kallistatin, especially in patients with NASH and HT.

Analysis of kallistatin levels by stages of liver steatosis revealed a significant decrease in kallistatin content with the progression of fatty liver parenchyma changes in patients with NASH and HT. A significantly higher content of kallistatin was found in patients with S1 steatosis (7.24 kPa (95% CI 6.67; 7.81), $P < 0.001$) and S2 steatosis (9.24 kPa (95% CI 8.46; 10.00), $P < 0.001$) in the group of patients with isolated NASH, significant differences were found only between the patients with steatosis at stages S1 (8.53 ng mL$^{-1}$) and S2 (9.04 ng mL$^{-1}$), $P < 0.001$).
(95% CI 84.42; 87.77)) and S2 (79.83 ng mL\(^{-1}\) (95% CI 78.21; 82.19); \(P < 0.001\)), while at the S3 steatosis stage the protein level decreased insignificantly and amounted to 75.43 ng mL\(^{-1}\) (95% CI 56.33; 101.49)).

Meanwhile, the group with isolated NASH showed a significant decrease in kallistatin in patients with S2 steatosis compared to S1 steatosis. Due to the insufficient number of participants in the isolated NASH patients with S3 steatosis (\(n = 3\)), it was impossible to confirm a significant change in the kallistatin level depending on progression of steatosis in those patients.

Ranking of patients with comorbidity of NASH and HT by liver fibrosis stages showed a significant kallistatin decrease with liver fibrosis progression from stage F0 to F3 (Table 2). Similar changes were observed only until F2 liver fibrosis in the group of patients with isolated NASH. It

\[\chi^2 = 6.723, P = 0.010\] 
\[\chi^2 = 1.9396, P < 0.001\] 
\[\chi^2 = 0.106, P = 0.745\] 
\[\chi^2 = 2.879, P = 0.090\]

Note: \(P < 0.05\) – the difference is statistically significant between groups.
indicates an additional negative effect of HT on the severity of kallistatin anti-inflammatory activity in late stages of liver fibrosis in NASH.

Statistically significant differences were found between the kallistatin levels in patients with NASH and HT depending on the hypertension stage. In HT I its content reached 71.82 ng mL$^{-1}$ (95% CI 70.16; 79.51), meanwhile in patients with HT II kallistatin levels averaged only 58.62 ng mL$^{-1}$ (95% CI 55.81; 64.45) ($P < 0.001$). The data obtained indicate a moderate inverse dependence of kallistatin activity on HT stage (rs = $-0.52$) and BP grade (rs = $-0.52$), strong and moderate inverse correlation with BP levels (rs = $-0.64$ and rs = $-0.43$ for SBP and DBP, respectively).

In addition, there were significant changes of indicator correlated with body mass index gain both in the group with NASH and HT (rs = $-0.52$), and in the group with isolated NASH (rs = $-0.54$).

In patients with NAFLD and HT with normal body weight, the level of the indicator averaged 72.86 ng mL$^{-1}$ (95% CI 56.07; 83.15), that was significantly higher than in the overweight patients, where the level of kallistatin decreased to 59.21 ng mL$^{-1}$ (95% CI 56.38; 64.52), $P < 0.001$. At the same time, the similar deviations were found in the group of patients with isolated NASH, where in patients with normal BMI the level of kallistatin averaged 86.37 ng mL$^{-1}$ (95% CI 83.35; 88.03), and in patients with increased body weight it was 80.76 ng mL$^{-1}$ (95% CI 79.49; 82.80), $P = 0.002$.

Kallistatin negatively correlated with ALT both in the main (rs = $-0.69$) and in the comparison group (rs = $-0.52$), as well as with AST (rs = $-0.55$ and rs = $-0.47$, respectively). Strength of the negative correlation between kallistatin and CRP in the group with NASH and HT (rs = $-0.84$) was significantly higher compared with isolated NASH group (rs = $-0.28$). Moderate correlations were found with the results of the NFS test in the main (rs = $-0.48$) and in the comparison group (rs = $-0.50$).

The absence of pronounced additional metabolic risk factors in the examined patients made it possible to exclude these analyzes from the study.

### Discussion

Various studies suggest that kallistatin concentrations vary in different chronic liver diseases, which may be associated with decreased hepatic protein secretion activity [25]. Cheng Z. et al. [26] found a significantly lower content of kallistatin in patients with liver fibrosis. Halla M. et al. [8] proved that even a single determination of the biomarker level allows to detect patients in the initial liver fibrosis stages with a sensitivity of 96.7% and a specificity of 50%. These data confirm the results of our study, where kallistatin levels differed between patients without fibrosis (F0) and with minor fibrosis (F1) both in the NAFLD and HT group ($P = 0.004$) and in the isolated NAFLD group ($p < 0.001$).
Prystupa A. et al. [19] determined that serum kallistatin levels decrease with the liver parenchymal damage progression in patients with alcoholic liver cirrhosis and higher kallistatin activity was observed in compensated cirrhosis patients compared to decompensated pathology cases (P < 0.05). Our results also indicate significant decrease in kallistatin levels depending on the liver fibrosis stage. This is explained by the antifibrotic kallistatin activity [12].

We found a significant kallistatin decrease with the liver parenchyma changes progression in NAFLD and HT patients. The literature data confirm that liver fat increase may lead to kallistatin production decrease due to the development of lipopolysaccharide inflammation [12]. It may be an evidence of kallistatin protection against liver parenchyma pathological changes [18].

Frühbeck, G. et al. [27] proved that kallistatin has a potential protective role in the obesity development in NAFLD. In our study, significant changes in kallistatin levels with weight gain in NAFLD patients were detected. This confirms the importance of the protein in protecting against metabolic adipose tissue changes in NAFLD.

Another important issue is the kallistatin role in the CVD. The hypertension development is associated with activity of the kallikrein-kinin system decrease [28]. Studies show that endogenous kallistatin is a protective agent against vascular oxidative stress, inflammation and fibrosis in animal hypertension models [12, 29]. The results of our study showed that kallistatin levels are significantly lower in patients with NAFLD and HT than in isolated NAFLD patients (P < 0.001).

The revealed inverse correlations of kallistatin and ALT, AST and CRP suggest that chronic systemic and local inflammatory processes reduce the activity of the anti-inflammatory marker.

However, literature data indicate a relatively low sensitivity (64%) and specificity (77%) of kallistatin as an indicator of liver cirrhosis [17]. In the detection of liver fibrotic changes in the study of Halla MR et al. [8] the kallistatin determination sensitivity was slightly higher and amounted to 96.7%, but the specificity of the test decreased to 50%. Simultaneous using non-invasive scales for liver fibrosis and steatosis assessment allowed to increase the sensitivity and specificity of kallistatin measurement to 90 and 76.8%, respectively. It can be considered sufficient for kallistatin determination as the biomarker for early liver fibrosis detection.

Thus, it is necessary to consider the possibility of combining the kallistatin determination with other non-invasive NAFLD tests, including the elastography and steatometry, which was one of the solutions in our study.

Conclusions

The presence of concomitant HT in patients with NAFLD is associated with significantly greater severity of fatty infiltration and fibrotic changes in the liver parenchyma, that was evidenced by a significant decrease in the incidence of S1 steatosis and F0 fibrosis and a corresponding increase in S3 steatosis and F2 fibrosis compared with isolated NAFLD.

The course of NAFLD is accompanied by a significant decrease in the content of kallistatin. Meanwhile, the comorbidity of NAFLD and HT leads to a statistically significant deepening of these deviations. The more pronounced steatosis and liver parenchymal fibrosis, activity of chronic systemic inflammation, increased BMI and higher HT stage may be considered as triggering factors for kallistatin activity inhibition.

The obtained data allow to consider kallistatin as a biomarker of NAFLD progression both in isolated NAFLD and in comorbidity of NAFLD and HT.

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ABBREVIATIONS

NAFLD non-alcoholic fatty liver disease
NASH non-alcoholic steatohepatitis
CVD cardiovascular diseases
HT arterial hypertension
BMI body mass index
BP blood pressure
SBP systolic blood pressure
DBP diastolic blood pressure
2D-SWE 2D shear wave elastography
AC attenuation coefficient
ROI region of interest
LS liver stiffness
NAS NAFLD activity score
ALT alanine aminotransferase
AST aspartate aminotransferase
CRP C-reactive protein
NFS Non-alcoholic Fatty Liver Disease Fibrosis Score

REFERENCES

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