Cholesterol, stearic and palmitic acids in the liver in nonalcoholic steatohepatitis of different severity according to the data of highly effective liquid chromatography-mass spectrometry

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Key words: Steatohepatitis, Cholesterol, Stearic Acid, Palmitic Acid, Chromato-mass Spectrometry.

It is known that higher saturated fatty acids (HSFA) having the lipotoxicity, play an important role in the development of non-alcoholic steatohepatitis (NASH), however, the results of research of cholesterol and HSFA in the liver with NASH of varying severity, as obtained by high performance liquid chromatography-mass spectrometry (HPLC-MS), have not been published yet.

The aim is to investigate the indicators of cholesterol, stearic and palmitic acids in the liver of the deceased patients suffering from non-alcoholic steatohepatitis with varying degrees of severity, and to compare them with indicators of blood plasma during the life of these patients.

Materials and methods. The conducted parallel posthumous pathomorphologic and HPLC-MS analysis of the liver of 25 patients, aged 53–88, who suffered during the life time of NASH and 10 deceased patients without the evidence of NASH, as well as a retrospective analysis of clinical and laboratory data in the medical records of these patients.

Results. It is found that the level of cholesterol in the liver increases from 0.42 (0.11; 4.49) mg/g to 7.10 (5.03; 8.25) mg/g (U=3.000; p=0.016) with increasing severity of NASH from S0 to S3. A statistically significant relationship between the content in the liver of stearic and palmitic acids takes place in patients with NASH (γ=0.681; p=0.001). Between the severity of NASH (y), as well as the elevated level of palmitic (x1) and stearic (x2) acids in the liver the significant relationship (γ=0.83x1; R2=0.33 and γ=1.13x2; R2=0.37) takes place.

Conclusions. In patients with the severe S3 mixed NASH peribiliar and intermediary zones of the liver lobules (LL) the significantly increased levels of stearic and palmitic acids in the liver were observed. In the severe combined total S3 NASH there is a statistically significant correlation between the severity of steatohepatitis (y), and cholesterol in the liver (x1) and cholesterol (x2) in the blood plasma (y=0.36x1+0.10x2; R2=0.91). In the severe S3 macrovesicular NASH of central and intermediate zones of LL a positive correlation between the elevated levels of total cholesterol in the blood plasma and high-density lipoproteins takes place.

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Вміст холестерину, стеаринової та пальмітинової кислот у печінці при неалкогольному стеатогепатиті різного ступеня тяжкості за даними високоєфективної рідинної хромато-мас-спектрометрії

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Відомо, насичені вищі жирні кислоти (НВЖК), що володіють ліпотоксичністю, відіграють важливу роль у розвитку неалкогольного стеатогепатиту (НАСГ), однак результати досліджень вмісту холестерину та НВЖК у печінці при НАСГ різного ступеня тяжкості, котрі одержані методами високоєфективної рідинної хромато-мас-спектрометрії (ВЕРХ-МС), поки не опублікувані.

Мета роботи – дослідити показники рівня холестерину, стеаринової та пальмітинової кислот у печінці померлих хворих, які страждали на неалкогольний стеатогепатит різного ступеня тяжкості, і порівняти їх із показниками плазми крові за життя цих пацієнтів.

Матеріали та методи. Здійснили паралельний посмертний патоморфологічний і ВЕРХ-МС аналіз печінки 25 померлих 53–88 років, які страждали за життя НАСГ, і 10 померлих пацієнтів без ознак НАСГ, а також ретроспективний аналіз клініко-лабораторних даних в історіях хвороби цих пацієнтів.

Результати. Встановлено, що зі збільшенням ступеня тяжкості НАСГ від S0 до S3 в печінці зростає рівень холестерину від 0.42 (0.11; 4.49) міл./г до 7.10 (5.03; 8.25) міл./г (U=3.000; p=0.016). У хворих НАСГ буває статистично значущий зв’язок між стеариновою (x1) та пальмітиновою кислотами (x2) в печінці відбувається вірогідна залежність (γ=0.83x1; R2=0.33 і γ=1.13x2; R2=0.37). У хворих із важким S3 змішаним НАСГ периферальних і проміжних зон печінкових часточок (ПЧ) у печінці вірогідно підвищений рівень стеаринової та пальмітинової кислот.

Висновки. При важкому S3 тотальному змішаному НАСГ відбувається статистично значуща залежність між ступенем тяжкості стеатогепатиту (y), а також рівнем холестерину в печінці (x1) і рівнем холестерину (x2) у плазмі крові (y=0.36x1+0.10x2; R2=0.91). При важкому S3 макрозвозикулярному НАСГ центральних і проміжних зон ПЧ тривається позитивна кореляція між підвищеним рівнем в плазмі крові загального холестерину й ліпопротеїнів високої щільності.

Ключові слова: стеатогепатит, холестерин, стеаринова кислота, пальмітинова кислота, хромато-мас-спектрометрія.

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Содержание холестерина, стеариновой и пальмитиновой кислот в печени при неалкогольном стеатогепатите разной степени тяжести по данным высокоэффективной жидкостной хромато-массспектрометрии

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Известно, что насыщенные высшие жирные кислоты (НВЖК), обладающие липотоксичностью, играют важную роль в развитии неалкогольного стеатогепатита (НАСГ), однако результаты исследований содержания холестерина и НВЖК в печени
Among the chronic liver disease non-alcoholic fatty liver disease (NAFLD) is very common now, which is a manifestation of the so-called dysmetabolic syndrome, including obesity, diabetes of the 2nd type, dyslipidemia and insulin resistance [1]. In the development of NAFLD nonalcoholic steatohepatitis with the benign course and nonalcoholic steatohepatitis (NASH), which is considered a precursor to the possible development of cirrhosis and liver cancer. Steatohepatitis transformation in NASH is associated with the phenomenon of lipotoxicity caused by the accumulation in the hepatocytes of higher saturated fatty acids (HSFA), which are an integral component of triglycerides (TG) [2]. Changing HSFA or their sequences in TG molecules increases their lipotoxicity promoting hepatocyte necrosis and apoptosis [3].

Experimental studies of S. Subramanian et al. [4] showed that the central molecules lipotoxicity with HSFA are free cholesterol and HSFA (eg, palmitic and stearic acid). In this connection, research on the content of cholesterol in the liver and HSFA in NASH of varying severity are of great interest.

K. Sumera (2013) [5] determined by gas chromatography in the liver tissue of patients with simple steatosis myristic (C14:0) and polyunsaturated γ-linolenic (18:3, ω-6) acids. In recent years, it is shown that in the development of NASH ω-6 eicosanoids play the significant role, which level in severe stages of steatosis and steatohepatitis in its transition. These arachidonic acid metabolites include 12-Hydroxyheptatrienoic acid (12-HHTRE) and 11,12-di-hydroxy-eicosatetraenoic acid (11,12-diHETE), and prostaglandins E2 and D2. Therefore, their plasma determination is offered to use as a noninvasive biomarker for differential diagnosis of NAFLD and NASH [6].

Today, the literature of the investigation results and total cholesterol and HSFA in the liver in NASH of varying severity, obtained by high performance liquid chromatography-mass spectrometry (HPLC-MS) is not available yet.

The purpose is to investigate the indicators of cholesterol, stearic and palmitic acids in the liver of the deceased patients suffering from non-alcoholic steatohepatitis with varying degrees of severity, and to compare them with indicators of blood plasma during the life of these patients.

Materials and methods
Conjunctive posthumous pathomorphologic and HPLC-MS analysis is held on the livers of 25 dead patients aged 53–88, who suffered from NASH during the life (first group of cases) and 10 patients who died without any microscopic evidence of NASH (second group of conditionally controlled cases), as well as a retrospective analysis of clinical and laboratory data in the case histories of these patients. NASH diagnosis was based on post-mortem histopathological examination of the liver and historical data in case histories of patients on the absence of alcohol abuse, the presence of overweight (BMI >25), diabetes of the 2nd type, duration of at least 1 year (glucose level of 5.5 mmol/l glycemic profile or glycohemoglobin >5.7 %) as well as data on the level of lipids in the blood plasma of patients. For the group of conditionally controlled patients the dead were selected, where no signs of steatohepatitis were detected at the histopathological examination of liver, and the lifetime performance and HSFA cholesterol in blood plasma did not exceed the reference values.

In patients who died, 2 samples of liver tissue were taken, one of them was fixated in 10% buffered formalin for pathological studies and the second one without any placing in formalin was used for HPLC-MS.

HPLC-MS analysis of tissue sample taken from the liver that was not fixated in formalin (200 mg) which was homogenised for 1 minute in Eppendorf adding isopropanol prior to homogenization and its following closure. Then the liver homogenate was incubated for 15 minutes in an ultrasonic bath Xuba 3 (“Grant Ltd”, UK) at a temperature 77 °C, then it had been centrifuged for 10 minutes at 15,000 rev/min in the centrifuge ULAB UC-1512 (China). Later the obtained supernatants were filtered by moving to the vial. Examination of cholesterol, stearic and palmitic acid was performed by the liquid-liquid extraction of the liver samples with the
subsequent HPLC-MS analysis of the obtained extracts on the instrument Agilent 1260 Infinity HPLC System (USA) and on single quadrupole mass spectrometer Agilent 6120 with ionization on electrospray (ESI) OpenLAB CDS Software using internal standards: cholesterol – fill. code (507363) S7509-STD concentration of 200 mg/dl (5.18 mmol/l) firms Pointe Scientific (USA), palmitic acid – fill. code (15104054) company Fluka (USA), puriss. p.a. standart for GC ≥98.5 % and stearic acid – fill. code (22303079) company Fluka (USA), puriss. p. a. standart for GC ≥99.5 %. During the HPLC-MS binary gradient was – eluent A: 60 % deionized H2O, 40 % CH3CN, 0.1 % HCOOH, 1.0 mM HCOONH4; eluent B: 10 % CH3CN, 90 % isopropyl alcohol, 0.1 % HCOOH 10 mM HCOONH4 (0 min – 32 % of eluent B, 5 min – 100 % eluent B, 10 minutes – 100 % eluent B); rate of eluent of 0.4 ml/min, 10 min of chromatography duration in the column Zorbax SB – C18; 30 mm x 4.6 mm; 1.8 microns with precolumn at a column temperature 55 °C, with electrospray ion source in the selective ion monitoring mode, cholesterol, stearic and palmitic acids (SIM) m/z 369, 283, 255 according to the monoisotopic mass of the protonated molecules at a 100, 150 and 150 V voltage on the defragmenter. The chromatological study was conducted at the positive polarity, the study of stearic and palmitic acids were conducted in the negative polarity. The temperature of nitrogen was 300 °C. Nebulizer pressure was at 60 psig. Gasdryer rate of nitrogen was 10 l/min. All data were recorded on the chromatograms.

Pathologic analysis. The presence of fatty degeneration of liver hepatocytes was evaluated in serial paraffin sections stained with hematoxylin and eosin, and also in parallel frozen sections stained with sudan III. The degree of NASH calculated by fotodigital morphometry serial paraffin sections stained with hematoxylin and eosin using digital medical image processing program ImageJ [7]. For this purpose, the pictures were taken in the microscope Axioplan 2 (CarlZeiss, Germany) by using of digital camera “Olympus 3040” (Japan) in each micropreparation of the liver to 5 fields of view at a magnification × 400. Further, the plug of Colour Deconvolution program ImageJ in the built-in analysis scheme, the obtained image was segmented and converted into binary and it was counted in % the number of pixels corresponding to the area of steatosis in hepatic lobules.

In accordance with gradation of E. M. Brunt, D. E. Kleiner (2005) [8] a mild S1 degree NASH was isolated (presence in a standardized field of view of the microscope 5 to 33 % of hepatocytes with micro- or macrovesicular steatosis), a moderate S2 degree (the presence of 34 to 66 % of the cells with a mostly macrovesicular or mixed steatosis) and a heavy S3 degree NASH (presence in the field of view of more than 66 % of hepatocytes with mixed steatosis). The absence of steatosis of hepatocytes in deceased patients of the second group of control observations was conditionally estimated as zero (S0) degree of hepatic steatosis.

The quantitative results of the studies were estimated using the statistical package Statistica® for Windows 6.0 (StatSoft Inc., № AXXR712D833214FAN5 license): the median of the lower and upper quartile was calculated, the data were presented in the form of Me (Q1, Q3). The level of cholesterol, palmitic and stearic acids in the liver were compared to the levels of total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG) in plasma and the degree of liver steatosis using coefficient-y. Using regression analysis the dependence of cholesterol levels of palmitic and stearic acids in the liver and content of total cholesterol was investigated and also LDL, HDL, TG in the blood plasma and the degree of NASH with the following statistical model constructing. The results were considered significant at p<0.05.

Results and its discussion

During integrated HPLC-MS and pathohistological analysis from deceased patients the significant differences between the level of cholesterol in the liver depending on the degree of hepatic steatosis were revealed (p<0.05). When comparing the degree of histological liver steatosis and cholesterol in liver indices, it was found that without hepatic steatosis (S0) the median level of cholesterol was 0.42 (0.11; 4.49) mg/g, and the liver with severe degree S3 steatosis – 7.10 (5.03; 8.25) mg/g (Fig. 1).

Thus, it was proved that the cholesterol in the liver without steatosis was significantly lower than in liver with the severe steatosis of S3 degree (U=3.000; p=0.016). The median of cholesterol level in the liver with a mild S1 degree of steatosis was 0.22 (0.08; 0.57) mg/g, which was significantly less than in a severe degree of steatosis S3 7.10 (5.03; 8.25) mg/g (U=1.000; p=0.005). The median level of cholesterol in the liver steatosis of moderate S2 degree 1.83 (0.41; 2.52) mg/g was significantly lower than in severe S3 grade of steatosis 7.10 (5.03; 8.25) mg/g (U=2.000; p=0.014). Median levels of cholesterol in the liver were not significantly different when compared to the control group with S0 steatosis with the groups of the mild S1 degree of steatosis (U=41.000;
p=0.744) and with moderate S2 degree (U=45.000; p=0.323), and also under the comparison between groups of S1 and S2 steatosis degree (U=27.000; p=0.055). According to P. Puriet et al. (2009) data, the level of free cholesterol in the liver was increased in patients with NASH and it remained unchanged in the healthy group of patients [9].

HPLC-MS analysis of the liver, in parallel with free cholesterol, cholesterol ester was also revealed (Fig. 2), which is capable to raise cholesterol in the liver tissue during the metabolic decay.

A retrospective analysis of laboratory data of patients with NASH was conducted and it showed that they had an elevated level of total cholesterol, LDL, HDL and TG in plasma during their lifetime (Table 1).

A. E. Feldstein [10] indicates a positive correlation between elevated TG, HDL, ALT and AST in the blood plasma of patients suffering from NASH.

In multivariate regression analysis between the chromatographic performance of cholesterol, stearic and palmitic acids in the liver and indicators of total cholesterol, LDL, HDL, TG in the blood plasma of patients who died with NASH, and patients who died without NASH the following features have been revealed: statistically the most significant was the relationship between the degree of hepatic steatosis (y), the level of cholesterol in the liver (x₁) and total cholesterol (x₂) in the blood plasma, where y=0.36x₁+0.10x₂; R²=0.91. In the statistical analysis of this model, its sensitivity was 88% for the comparison of the control group with the NASH group (regardless of its degree), and under the delimitation of NASH into groups according to the steatosis degree it was 68%. The specificity of the model was 70%. A comparative analysis of the cholesterol level in the blood plasma in patients and the severity of NASH demonstrated that elevated level of total cholesterol in blood plasma, and elevated levels of cholesterol in the liver corresponds to the severe S3 total mixed steatosis of hepatocytes of central, intermediate and perportal areas of the liver lobules (Fig. 3) which was established in postmortem liver pathohistological study.

According to the literature data the process of free cholesterol metabolic dysregulation in patients with NASH is caused by presence of the 2nd type of diabetes and insulin resistance, in which the synthesis of cholesterol is increased and its excretion from the liver through the efflux pathway is reduced [11,12].

When analyzing the interrelations between severity of NASH and the Lipidogram data of patients a statistically significant positive correlation between the content of total cholesterol and HDL cholesterol in the blood plasma and

### Table 1

| The severity of NASH | Total cholesterol | LDL | HDL | TG |
|----------------------|-------------------|-----|-----|----|
|                      | Me     | Q1  | Q3  | Me | Q1  | Q3 | Me | Q1  | Q3 |
| S0                   | 4.19   | 3.79| 5.30| 3.67| 2.92| 4  | 1.11| 0.78| 1.75| 1.12| 0.77| 1.7 |
| S1                   | 4.47   | 4.18| 4.18| 2.85| 2.27| 4.65| 1.01| 0.78| 1.75| 1.12| 0.77| 1.7 |
| S2                   | 5.25   | 3.93| 5.82| 3.71| 2.59| 4.81| 1   | 0.77| 1.56| 1.18| 0.75| 1.56|
| S3                   | 4.44   | 3.79| 6.21| 2.77| 1.97| 6.09| 0.93| 0.63| 1.13| 1.70| 0.93| 2.30|

Notes: LDL – low density lipoprotein, HDL – high-density lipoprotein; TG – triglycerides, Me – mediana, Q1 – upper quartile, the Q3 – bottom quartile.

![Fig. 2. The chromatograms of cholesterol at 2 SIM m/z 369. A – in the control group without liver steatosis S0. B is the group of patients with the severe S3 degree of NASH. The cholesterol peak is indicated with the red arrow, the cholesterol ester peak is indicated by the green arrow.](image-url)
between an increased NASH degree and increased total cholesterol and HDL cholesterol in the blood plasma was revealed (Table 2). When comparing the severity of NASH only with indicators of HDL levels a negative relationship was observed: with the increase of NASH severity a decrease in HDL levels in the blood plasma was observed (Table 2).

The heavy S3 macrovesicular steatosis of hepatocytes of central and intermediate zones of the liver lobules was revealed in patients with elevated levels of total cholesterol and HDL cholesterol in blood plasma of the post-mortem pathological examination of the liver (Fig. 4).

Under the correlation analysis of HSFA in the liver of patients with NASH a statistically significant relationship between the content of stearic (C18:0) and palmitic acids in the liver is revealed (C16:0), \( \gamma=0.681; p=0.001 \). At higher levels of palmitic acid in the liver, the level of stearic acid also increases (Fig. 5).

In histopathological examination of the liver in patients who died of this group the heavy S3 mixed steatosis of hepatocytes in the perportal and intermediary zones of the liver lobules was revealed (Fig. 6).

In NASH the elongase enzyme is activated in damaged hepatocytes as well as the elongation process of palmitic and stearic acids, i.e. at excessive increased levels of palmitic acid in the liver stearic acid is synthesized from it, which has greater lipotoxicity to the liver. The increase of HSFA in blood plasma results in reduced insulin secretion of pancreatic \( \beta \)-cells [13], in activation of the enzyme of terminal kinases (JNKs) and protein FoxO3a [11,14], which are involved in hepatocytes lipoapoptosis.

The HPLC-MS performed studies have shown that the level of palmitic and stearic acids is increased in patients with severe liver NASH, compared to their content in the liver of patients without NASH (Fig. 7, 8).

Under the paired regression analysis statistically most significant relationship between the level of NASH \( y \) and the level of palmitic \( x_1 \) and stearic \( x_2 \) acids in the liver is revealed, where \( y=0.83x_1; R^2=0.33 \) and \( y=1.13x_2; R^2=0.37 \). According to P. Puri data [9] the HSFA levels in the blood plasma of patients with NASH are elevated, which excessive deposition in the liver can lead to the destruction of hepatocytes with peroxisome. A. E. Feldstein [10] describes the increase of fatty acid levels in blood plasma at early stages of NASH which is caused by monounsaturated palmitoleic acid (C16: 1n7), and indicates that further increase of fatty acid level in plasma and launch of processes of their elongation leads to an increase of the saturated palmitic and stearic acids levels in the liver.

According to the data of A. Takaki et. al. [15], Y. Takeshita et. al. [16] the excessive concentration of free cholesterol and HSFA in the liver leads to disruption of mitochondrial function, and the endoplasmic reticulum, it also activates a cascade of oxidative stress and intracellular accumulation of oxysterols that have lipotoxic effect and lead to fatty degeneration of hepatocytes. With the progression of

### Table 2

| Parameters                      | Total cholesterol of blood plasma (mmol/l) | HDL (mmol/l) | Histological degree of NASH |
|--------------------------------|------------------------------------------|--------------|----------------------------|
| Total cholesterol of blood plasma (mmol/l) | 1            | –            | –                          |
| HDL (mmol/l)                  | 0.54*         | 1            | –                          |
| Histological degree of NASH   | 0.18*         | -0.23**      | 1                          |

Notes: The data were analyzed using regression analysis. * – the positive correlation between the histologic NASH degree and indicators of total cholesterol to high-density lipoproteins in blood plasma; ** – the negative correlation between the histologic NASH degree and indicators of high density lipoproteins in blood plasma.
Fig. 5. The correlation between the content of palmitic and stearic acids in the liver of patients with NASH.

Fig. 6. The heavy S3 mixed steatosis of hepatocytes in periportal and intermediary zones of hepatic lobules. H & E stain. Magnification: x200.

Fig. 7. Chromatograms of palmitic acid at SIM m/z 255. A is in the control group without liver steatosis (S0). B is in the liver of patients group with the severe S3 degree of NASH. The palmitic acid peak is indicated by the arrow.

Fig. 8. The chromatograms of stearic acid in SIM m/z 283. A is in the control group without liver steatosis (S0). B is in the liver of patients group with the severe S3 degree of NASH. The stearic acid peak is indicated by the arrow.
NASH the damaged hepatocytes activate the macrophage NALP3-inflammasome which promotes the aggregation of Kupffer macrophages around the outbreaks of lipoapoptosis hepatocytes and they also activate stellate cells of perisinusoidal fibrogenic phenotype with the subsequent formation of liver fibrosis and cirrhosis [17].

Conclusions
1. The comparative analysis of histopathological and chromatography-mass spectrometry data showed a significant increase of cholesterol level in the liver by increasing of the steatohepatitis severity: in the S0 steatohepatitis the cholesterol level in the liver is 0.42 (0.11; 4.49) mg/g, in the S3 steatohepatitis it is 7.10 (5.03; 8.25) mg/g (U=3.000; p=0.016).
2. In the severe combined total S3 nonalcoholic steatohepatitis of central, intermediate and periportal regions of the hepatic lobules there is a positive correlation between elevated levels of total cholesterol in the blood plasma and high-density lipoprotein.
3. In the severe S3 macrovesicular nonalcoholic steatohepatitis of the central and intermediate zones of the hepatic lobules there is a positive correlation between elevated levels of total cholesterol in the blood plasma and high-density lipoprotein.
4. Between the severity of nonalcoholic steatohepatitis (y), as well as elevated levels of palmitic (x1) and stearic (x2) acids the significant relationship takes place in the liver (y=0.83x1; R²=0.33 and y=1.13x2; R²=0.37).
5. In patients with non-alcoholic steatohepatitis there is a statistically significant relationship between the content in the liver of stearic and palmitic acids (γ=0.681; p=0.001).
6. In patients with severe S3 mixed nonalcoholic steatohepatitis in the periportal and intermediary zones of hepatic lobules the significantly increased levels of stearic and palmitic acids take place in the liver.

Prospects for further research. The further chromatography-mass spectrometry study of HSFA content in the liver in alcoholic and nonalcoholic steatohepatitis of varying severity is of considerable interest.

Conflicts of Interest: authors have no conflict of interest to declare.

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