MEDICAL UNIVERSITY

"PROF. DR. PARASKEV STOYANOV", VARNA

II DEPARTMENT OF INTERNAL DISEASES

DEPARTMENT OF GASTROENTEROLOGY, HEPATOLOGY AND NUTRITION

DR. PAVLINA GEORGIEVA BOYKOVA-VALCHEVA

SCREENING, DIAGNOSIS AND CLINICAL EVALUATION OF PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

ABSTRACT

ON DISSERTATION PAPER

FOR THE AWARD OF THE SCIENTIFIC AND EDUCATIONAL DEGREE "DOCTOR"

Scientific specialty "gastroenterology"

Supervisor

Prof. Dr. Iskren Andreev Kotsev, MD

Scientific jury:

Prof. Dr. Ivaylo Petrov Vazharov, MD

Assoc. Dr. Vladimir Nikolov Andonov

Assoc. Dr. Miglena Slavcheva Stamboliyska-Nikolova, MD

Assoc. Dr. Irina Ivanova Ivanova, MD,

Assoc. Dr. Diana Todorova Gancheva-Tomova, MD

Varna, 2022

The dissertation contains 157 pages and is illustrated with 50 figures and 58 tables. The cited literature includes the sources from which Cyrillic and Latin.

The dissertation was discussed and referred for public defense by the departmental council of the II Department of Internal Medicine of the Medical University - Varna on July 6, 2022.

The official defense of the dissertation will take place on October 19, 2022 at the open meeting of the Scientific Jury.

CONTENTS

Most commonly used abbreviations:

AASLD – American Association for the Study of Liver Diseases

APRI - AST to Platelet Ratio Index

ALT- alanine aminotransferase

AST – aspartate aminotransferase

AUROC - Area Under the Receiver Operating Characteristic curve

BMI – Body mass index

CAP – controlled attenuation parameter

CRP – C-reactive protein

DM – diabetes mellitus

EASD - European Association for the Study of Diabetes

EASL - European Association for the Study of the Liver

EASO- European Association for the Study of Obesity

GGT – gamaglutamiltransferaze

HDL – high density lipoproteins

HD - Hypertensive Heart Disease

HOMA-IR - Homeostasis Model Assessment – Insulin Resistance

FAST score – Fibroscan – AST score

FIB-4 – fibrosis 4 index

LDL – low density lipoproteins

MAFLD – Metabolic associated fatty liver disease

MRI – magnetic resonance immaging

NAFLD – Nonalcoholic fatty liver disease

NASH – Nonalcoholic steatohepatitis

NFS-NAFLD fibrosis scor

PNPLA3 – Patatin-like phospholipase domain-containing protein 3

TE – transient elastography

u.l.n. – upper limit of normal

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a public health problem of global importance, affecting approximately one million individuals worldwide. NAFLD 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. NAFLD is divided into two main groups: Nonalcoholic steatosis of the liver (NASL) and Nonalcoholic steato hepatitis (NASH). NASH 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 - nonalcoholic steatosis hepatitis is characterized histologically by the presence of inflammation and hepatocyte balloon degeneration, which determines the faster progression of the disease.

NAFLD arises from the interaction of environmental, epigenetic and genetic factors and is directly related to the presence of insulin resistance (IR). There is a bidirectional relationship between the metabolic syndrome and NAFLD. The metabolic syndrome and its components predict the development and progression of NAFLD, but the presence of NAFLD is a precursor for future occurrence of the components of the metabolic syndrome. NAFLD is associated with the presence of obesity, type 2 diabetes mellitus, hypertension, and metabolic syndrome. Because of the proven association of NAFLD with the metabolic syndrome, it has been proposed by an international panel of experts to replace the term non-alcoholic fatty liver disease with the term metabolic-associated fatty liver disease (MAFLD). 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. Advanced fibrosis is recognized as a major factor predicting adverse outcome and mortality in patients with NAFLD. Therefore, distinguishing NASH from pure hepatic steatosis and determining the presence of advanced liver fibrosis is of great importance in patients with NAFLD. Considering the huge number of patients at risk, it is of great importance to perform screening and clinical evaluation of patients with NAFLD, regarding the risk of hepatitis and advanced fibrosis. Still the gold standard for making the diagnosis of NASH and determining the stage of fibrosis in patients with NAFLD is histological examination. Liver biopsy has its well-known limitations, such as invasiveness, risk of complications, variability of histological evaluation by different experts, sampling errors, impossibility of dynamic follow-up, patient refusal to perform biopsy. As a result of these disadvantages of liver biopsy, alternative non-invasive biomarkers to identify patients with non-alcoholic steatohepatitis and advanced fibrosis from the general group of NAFLD patients have been intensively investigated and used over the past decade. Various non-invasive methods are used, including serum biomarkers and imaging techniques – ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and elastography-based ultrasound methods, one of

which is transient elastography (TE). All non-invasive methods for NAFLD staging are still under investigation and research, and a universally accepted world standard is lacking.

NAFLD presents many unsolved problems and questions, both in terms of pathogenesis and clinical aspect in terms of diagnosis, follow-up, clinical evaluation, treatment and need for screening.

2. Purpose and tasks:

2.1. Purpose:

With the present work, we aimed to study the issues related to screening, diagnosis and clinical evaluation in patients with NAFLD.

2.2. Tasks:

To fulfill the above-mentioned aim, we have set ourselves the following tasks:

1. To clarify the role of clinical examination and ultrasound examination in patients with NAFLD, by analyzing the clinical evaluation of patients, based on which to determine a correlation between anthropometric parameters and the established degrees of steatosis and fibrosis.
2. To elucidate the role of controlled attenuation parameter (CAP) measurements for the clinical assessment of the degree of steatosis in patients with NAFLD and seek correlation with established ultrasonographic steatosis and defined steatosis indices.
3. To clarify the role of fibroscan elastography to assess the stage of fibrosis in patients with NAFLD.
4. To analyze the application of cytokeratin 18 as a diagnostic marker of inflammation in NAFLD and its relationship with anthropometric parameters, routine laboratory tests, ultrasonographically determined degree of steatosis and stage of fibrosis determined from non-invasive scores.
5. To investigate the correlation between non-invasive serum and imaging methods for staging steatosis and fibrosis.
6. To clarify the role of applied non-invasive methods (serum biomarkers and transient elastography) as a screening to detect patients with NAFLD and the possibility to stratify the risk groups for presence of advanced disease.
7. To propose an algorithm for screening and diagnosis of patients with NAFLD.

3. Material and methods.

3.1. Subjects studied.

A total of 148 persons were included in the study, examined at the Gastroenterology Clinic at UMHAT ‘’Saint Marina’’ - Varna for the period from October 2016 to May 2022. Patients admitted to the clinic due to elevated transaminases and suspected NAFLD were included, patients admitted in the clinic on another occasion, but with evidence of hepatic steatosis, patients who visited the clinic's Gastroenterology consultation office and with evidence of hepatic steatosis, patients hospitalized in the Endocrinology Clinic with evidence of metabolic syndrome and/or type 2 DM , which were actively screened. All patients (n=148) had evidence of nonalcoholic fatty liver disease, 38 patients were diagnosed with nonalcoholic steatohepatitis, 100 patients with nonalcoholic steatosis, and 10 with compensated cirrhosis (8 with inactive cirrhosis and 2 with active cirrhosis). There was no patient with hepatocellular carcinoma in our study group.

In 91 patients, in addition to a complete clinical examination, laboratory and ultrasound examination, elastography was performed, with determination of CAP on a FibroScan, Echosence device for the period from October 2016 to July 2019. In 61 patients, a complete clinical and ultrasound examination was performed and in addition laboratory tests including cytokeratin 18 for the period June 2021 to May 2022.

The mean age of the studied patients was 55.68 years, with a minimum age of 23 years and a maximum age of 82 years. Of them 65 were men and 83 women. The largest number (n=15) of men were from the age group 50-59 years, followed by the interval - 40-49 years (n=14) and the same number in the interval 60-69 years (n=14), 11 were in the interval – 30-39 years, 6 - in the age group 70-79 years, and 5 in the interval 20-29 years. There were no men in the age group over 80 years. The largest number of women (n=29) were from the 50-59 age group, followed by 24 women - from the 60-69 age group, 17 women - in the 70-79 age range, 6 women - in the 40 age range -49 years, 5 – in the interval 30-39 years, 1 – from 20 to 29 years and 1 patient over 80 years.

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 heart failure III-IV functional class 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. The results of the observations and the clinical indicators of the patients were systematized in tabular form with a view to optimizing the processing.

3.2. Methodes

The following studies were conducted:

3.2.1. Medical history and objective physical examination

A detailed medical history of subjective complaints, accompanying diseases, medications taken and harmful habits was taken from the patients. Only patients who did not have excessive alcohol use according to the standard were included. Calculations for the daily consumption of absolute alcohol are made on the basis of recalculation through 1 standard alcohol unit, which is equal to about 10-14 g of absolute alcohol according to the formula: 1 alcohol unit = amount of alcohol (ml) x concentration (%)/ 1000. One alcohol unit is contained in 25-30 ml of concentrated (40%) alcoholic drink (whiskey, vodka, brandy), 100-120 ml of wine or in 250 ml of beer. The presence of arterial hypertension, dyslipidemia, diabetes mellitus, family history of metabolic disorders was specified.

Anamnestically, patients rarely report complaints. In our study group (N=148), 9 patients reported heaviness and discomfort in the right lower quadrant with a feeling of prop and swelling. In 32 patients, steatosis was detected during a prophylactic ultrasound examination or during an ultrasound performed because of abdominal pain, upper or lower dyspeptic syndrome associated with another pathology. In 20 patients, steatosis was proven due to established elevated transaminases or cholestasis enzymes, during preventive laboratory tests or tests on another occasion. In most of the patients in our group, steatosis was detected during preventive examination and targeted screening among patients with evidence of metabolic syndrome. In a part of all these patients, advanced and active liver disease is established in opposition to the absence of complaints and the lack of awareness of the presence of an existing liver problem.

A complete physical examination was performed including measurement of anthropometric parameters - height (cm) and body mass (kg). Based on these data, a body mass index (BMI) in kg/m² (weight in kilograms divided by height in meters squared) was calculated. BMI is a widely accepted method for defining and classifying body mass in individuals over 18 years of age. The values are age-independent and the same for both sexes.. Normal weight is accepted at a BMI between 18.5 kg/m² - 24.9 kg/m², overweight at BMI 25.0 kg/m² – 29.9 kg/m², obesity I degree at BMI 30.0 kg/m² – 34.9 kg/m², obesity II degree at BMI 35.0 kg/m² – 39.9 kg /m² and obesity III degree at BMI ≥ 40 kg/m², according to WHO 2000 and the European Association for the Study of Obesity.

Waist circumference was another anthropometric parameter that was measured in patients. It is measured midway between the lower edge of the costal arch (lower border of the 10th rib) and the upper edge of the iliac crest at the end of a normal calm expiration (WHO 2008). Waist circumference is a good anthropometric indicator of visceral fat accumulation. It is gender and ethnically stratified. The upper limit of the norm above which the presence of visceral obesity is accepted for the Caucasian race is ≥ 94 cm for men and ≥ 80 cm for women.

According to the WHO, two levels of abdominal obesity are defined related to the degree of risk of metabolic disorders and health problems, presented in the following table (table 1). Increased waist circumference may predict increased health risk, even with a normal BMI.

	Increased risk	Significantly increased risk
Men, waist circumference	≥ 94 cm	≥ 102 cm
Women, waist circumference	≥ 80 cm	≥ 88 cm

Table. 1 levels of abdominal obesity

3.2.2. Standard laboratory tests.

The tests were performed 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%. In patients with elevated transaminases, serum copper and ceruloplasmin were examined to differentiate Wilson's disease. The tests were carried out in the Central Clinical Laboratory of UMHAT "St. Marina", Varna.

In patients with elevated transaminases were performed 1) virological tests, using various commercial ELISA kits to detect HBsAg, anti-HCV, anti-HDV, anti HBcor total with a view to exclud chronic viral hepatitis, 2) immunological tests - autoantibodies (AMA, ANA, anti SMA, anti LKM, pANCA, cANCA) with the methods of indirect immunofluorescence and Westernblot (immunoblot), to exclude autoimmune liver disease.

To admit the diagnosis of metabolic syndrome, we used the criteria of the International Diabetes Federation (IDF), according to which MS is accepted in the presence of at least 3 of the following 5 criteria:

1. Abdominal obesity (waist circumference ≥ 94 cm for men and ≥ 80 cm for women);

2. Elevated serum triglycerides (> 1.7 mmol/l) or specific treatment for this lipid disorder;

3. Reduced values of HDL-cholesterol in the serum (< 1.0 mmol/l for men and < 1.3 mmol/l for women) or ongoing treatment for dyslipidemia;

4. Arterial pressure > 130 mm Hg for the systolic or >85 mm Hg for the diastolic, or taking antihypertensive medications for the treatment of hypertensive disease;

5. Fasting plasma glucose ≥ 5.6 mmol/l or type 2 DM treatment.

In the patients included in the study, glucose metabolism was examined and whether they met the criteria for metabolic syndrome was assessed. In some of them, HOMA-IR was examined to prove insulin resistance.

The assessment of glucose metabolism disorder is consistent with accepted clear definitions. Normal fasting glycemia/normal glucose tolerance are respectively accepted at plasma glucose lower than 5.6 mmol/l and plasma glucose at the 2nd hour of oral glucose loading - lower than 7.8 mmol/l, HbA1c < 5.7 mmol/l. Impaired fasting glycemia is defined as fasting plasma glucose equal to or higher than 5.6 mmol/l and lower than 7.0 mmol/l. Impaired glucose tolerance - plasma glucose on the 2nd hour after oral glucose load equal to or higher than 7.8 mmol/l, but lower than 11.1 mmol/l; HbA1c < 6.5% and ≥ 5.7%. Impaired fasting glycemia and impaired glucose tolerance are considered prediabetes according to the

ADA (American Diabetes Association) criteria. Diabetes mellitus is accepted when fasting plasma glucose is ≥ 7.0 mmol/l or at the second hour of oral glucose loading ≥ 11.1 mmol/l, or random plasma glucose ≥ 11.1 mmol/l and HbA1c > 6.5%.

To assess insulin resistance, we used the calculation of HOMA-IR (Homeostasis Model Assessment – Insulin Resistance), calculated from the values of fasting glucose (mmol/l) and fasting insulin (mU/l), according to the following formula - HOMA-IR = Insulin fasting x glucose /22.5. We considered insulin resistance at values of HOMA-IR ≥2.5.

3.2.3. Determination of the value of cytokeratin 18

Cytokeratin 18 (CK18) in serum was studied in 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 CK18 in the serum by "sandwich" ELISA method, which uses specific antibodies - anti-Human Cytokeratin 18 (Millipore, product number RAB1408). After physical examination and ultrasound examination, venous blood was taken from the patients. The serum was separated from the collected blood, after which all samples were stored at a temperature below -20°C until the time of analysis. The measurement units of the obtained results are in ng/ml.

3.2.4. Non-invasive predictive mathematical models - score systems

Using some laboratory parameters and clinical characteristics of the patients, the following scores were calculated to assess steatosis, the presence of hepatitis, and fibrosis.

A. Calculated scores for steatosis assessment:

- *HSI – Hepatic steatosis index* – it was calculated based on ALT, AST, BMI, gender, presence of DM. It is calculated according to the formula: hepatic steatosis index (HSI)= 8 x (ALT/AST ratio)+BMI (+2 if female; +2 if type 2 DM). Values <30.0 are considered to rule out NAFLD, and values >36.0, HSI establishes the presence of NAFLD. The HSI is a simple and efficient screening tool for NAFLD that can be used to select individuals for liver ultrasound and to determine the need for lifestyle modification.

Withouth steatosis	<30	HSI >36	With steatosis

- *LAP – Lipid Accumulation Product* – it was calculated based on waist circumference, Tg, sex. It is an easily accessible and inexpensive index predicting the presence of MS, insulin resistance, and also has a proven predictive value for the onset of DM and determining the degree of cardiovascular risk. This index presents the presence of central obesity. It is suitable for screening for MS. Recent studies have shown a very high predictive value for the presence of NAFLD.

It is calculated by the formula: LAP = (waist circumference [cm] − 65) × (triglyceride concentration [mmol/l]) for men, and (waist circumference [cm] − 58) × (triglyceride concentration [mmol/l]) for women. The suggested cut-offs vary depending on nationality,

and according to some authors, on gender and age. Suggested test validation values are: a cut-off value of LAP <20 rejects steatosis, while a value of LAP ≥80 accepts steatosis.

Without steatosis	<20	LAP	≥80	With steatosis

- *Fatty liver index (FLI)* – was calculated based on BMI, waist circumference, Tg level and GGT according to the formula:

$$FLI = \left(e^{(0.953\times\log e(Tg)+0.139\times BMI+0.718\times\log e(ГГТ)+0.053\times талия-15.745}\right)/ \left(1 + e^{0.953\times\log e(Tg)+0.139\times BMI+0.718\times\log e(GGT)+0.053\times талия-15.745}\right) \times 100.$$

Index values range from 0 to 100. FLI below 30 – (negative predictive value = 0.2) rules out steatosis; FLI greater than or equal to 60 – (positive predictive value = 4.3) proves steatosis. FLI is a simple and accurate index for predicting the presence of steatosis. Convenient for selecting patients for ultrasound examination and the need for consultation to change lifestyle. Predicts metabolic and cardiovascular risk, liver and cardiovascular morbidity.

Without steatosis	<30	FLI	≥60	With steatosis

- *NAFLD liver fat score* was calculated based on the presence of DM, serum insulin, AST, AST/ALT ratio according to the formula: 1.18× metabolic syndrome +0.45× DM (2, if yes; 0, if no) +0.15× fasting insulin (mU/ L) +0.04× ASAT (U/L) −0.94× (ASAT/ALT) − 2.89. The optimal cut-off value is > -0.64 with sensitivity 86%, specificity 71%.

B. A calculated predictive model for evaluating the presence of steatohepatitis is the FibroScan-AST score (FAST score). Includes TE-determined liver density, CAP and AST value. It was developed in 2020. FAST score is a mathematical model for non-invasive identification of NASH patients with significant fibrosis and activity. Therefore, this score provides an effective non-invasive way to identify patients at risk of progressive disease. The AUROC ranged from 0.74 to 0.95, with a positive predictive value of 0.83 and a negative predictive value of 0.85. A value ≤0.35 excludes significant inflammation and fibrosis. A value of 0.35-0.67 indicates active disease, with significant fibrosis (NASH + NAS≥4 + F≥2), and a value above ≥ 0.67 is associated with advanced disease.

C. Calculated scores for non-invasive assessment of fibrosis:

- *APRI скор (AST to **P**latelet **R**atio **I**ndex - APRI)* calculated based on the upper reference value of ASAT and platelet count according to the following formula:

$$\text{APRI} = \frac{\text{AST level (/ULN)}}{\text{Platelet count } (10^9/\text{L})} \text{ X } 100$$

Values < 0.5 reject the presence of fibrosis, > 0.7 - suggest advanced fibrosis (F3 - F4), and value > 1 - cirrhosis (F4). Cirrhosis is certain with an APRI score > 2, but with lower specificity.

- *Fibrois-4 (FIB-4*) was calculated based on the values of age, AST, ALT and platelets according to the following formula: FIB-4 = (Age x AST) / (Platelets x (√ (ALT).

FIB-4 < 1.30 rejects advanced fibrosis (F3-F4), FIB-4 between 1.30 and 2.67 – gray zone, and FIB-4 > 2.67 predicts advanced fibrosis (F3 – F4) (table. 15).

- *NFS (NAFLD fibrosis score)* was calculated based on the values of age, BMI, increased fasting glycemia or DM2, AST, ALT, platelets and albumin according to the following formula: NAFLD fibrosis score = -1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m2) + 1.13 × impaired fasting glycemia/DM (yes = 1, no = 0) + 0.99 × AST/ALT - 0.013 × platelets (×109/L) - 0.66 × albumin (g/dL).

NFS <-1,455 – rejects significant fibrosis (F0-F2), NFS between ≤-1,455 and ≤ 0.675 – gray zone, NFS >0,675 - predictor of significant fibrosis (F3-F4) (table 15).

- *BARD* score was calculated based on BMI, ACAT/ALT and the presence of DM as follows: ratio ACAT/ALT ≥0.8 – 2 points; BMI ≥28 – 1 point; and presence of DM type 2 – 1 point. The possible number of points ranges from 0 to 4. The presence of 2 factors is associated with a 17-fold increase in the risk of advanced fibrosis. According to the results of Harrison and co-authors, BARD score 0 and 1 has a negative predictive value of 96% for advanced fibrosis (Table 15).

- *AST/ALT ratio* – based on literature data, we assumed that values above 1 are predictive of advanced fibrosis. The AST/ALT ratio is used in the rest of the rate systems. It is also a predictor of alcohol use (Table 2).

All of the above formulas of the predictive models were entered into the Excel spreadsheet using a programmer and were automatically calculated after entering the relevant data for each individual patient.

APRI	NAFLD score	fibrosis	FIB-4 score	AST/ALT
< 0.5	< -1.455	**F0 -F2**	< 1,3	<0,8
0,5 ÷ 0,7	-1,455 ÷ 0,675	**Indeterminate score**	1,3 ÷2,67	0,8÷1
> 0.7	>0,675	**F3-F4**	>2,67	>1

Table 2 Threshold values for APRI, NAFLD fibrosis score, FIB-4, AST/ALT

3.2.5. Abdominal ultrasound

All patients underwent a standard ultrasound examination of the abdominal organs with an Aloka Prosound alfa7 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 hollow organs. The following criteria for steatosis were used (Fig. 6):

0 – normal echogenicity;

1 st - mild steatosis, slight diffuse increase in the echogenicity of the liver parenchyma, compared with the parenchyma of the right kidney, with normal visualization of the diaphragm, intrahepatic vessels and the gallbladder wall;

2 st – moderate steatosis, moderate diffuse increase in the echogenicity of the liver parenchyma, compared with the parenchyma of the right kidney, with slightly impaired visualization of the intrahepatic vessels, the diaphragm and the gallbladder wall;

3 st - severe steatosis, significantly increased echogenicity, relative to the parenchyma of the right kidney, with poor or absent visualization of the intrahepatic vessels, the diaphragm, the posterior segments of the right lobe, and the gallbladder wall.

Anterior-posterior liver size was also measured and focal sparing was assessed. The surface and structure of the liver was described, its elasticity was assessed and the presence of data on portal hypertension - diameter of portal vessels, splenomegaly and ascites. Longitudinal, transverse size of the spleen and its area were measured.

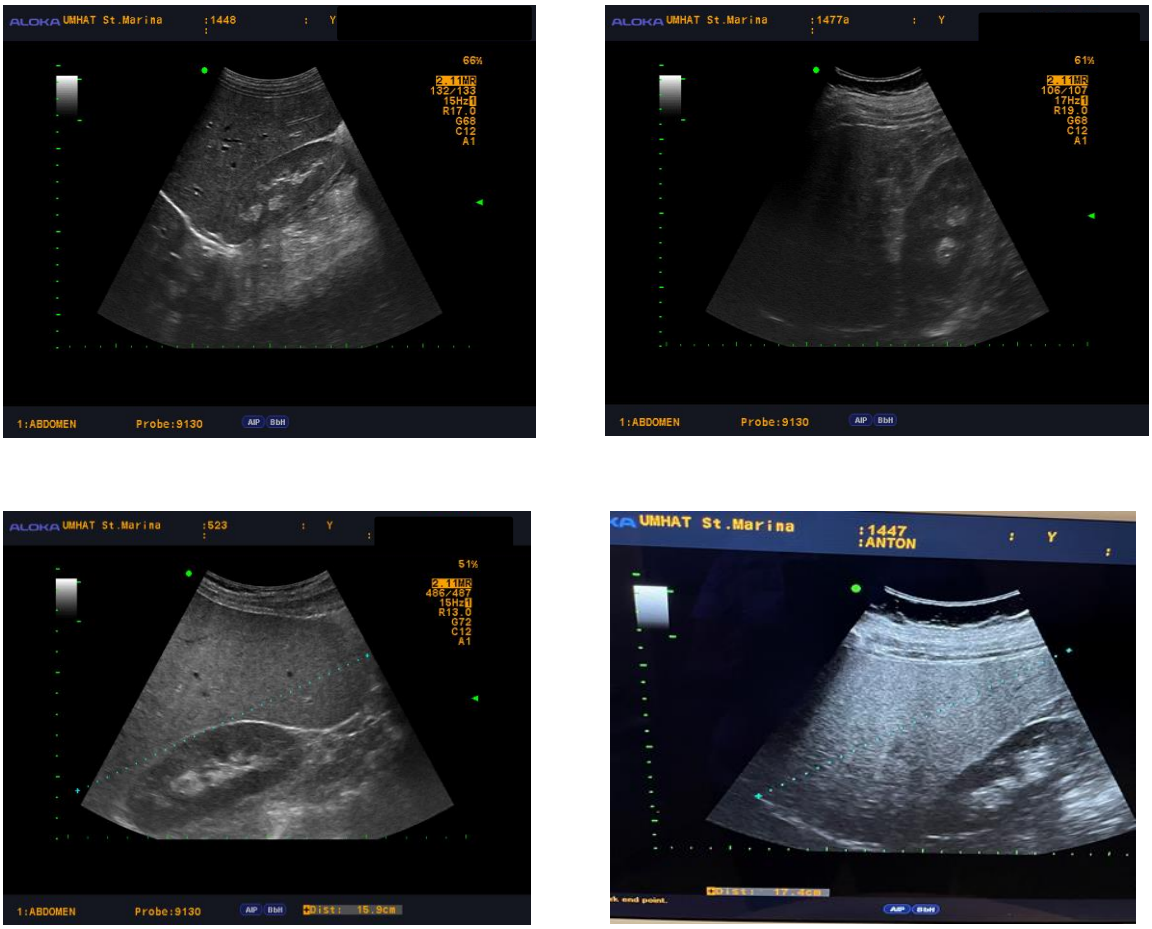


Fig. 1 Ultrasound images of different degrees of hepatic steatosis of patients participating in the study

3.2.6. Transient elastography

A FibroScan 502 device with two M probes and an XL probe, manufactured by Echosens, France, was used to determine Liver stiffness (LS) and CAP (Controlled Attenuation Parameter) for quantitative measurement of steatosis. The CAP software was installed only on the M probe.

Transient elastography was performed in 91 patients (45 men and 46 women) with a mean value and variability interval of at least 10 valid measurements. The mean age of the

patients was 55.27 years, ranging from 23 to 82 years. The examinations were performed according to the requirements of fasting, with at least 2 hours before the patients did not take food. The patient's position is lying on his back with his right arm raised above his head and his body bent slightly to the left side. Measurements are made in the right lobe of the liver, intercostally, with the transducer placed perpendicular to the liver capsule. Liver density in kPa representing the stage of fibrosis and CAP in dB/m were measured simultaneously to quantify the degree of steatosis.

Assessment of fibrosis stage from 0 to 4 was performed according to accepted cut-offs from meta-analyses of studies in patients with NAFLD and NASH. For normal elasticity we take values up to 5-5.5 kPa, fibrosis stage F1 is taken up to 7 kPa, fibrosis stage F2 is taken up to 10 kPa, fibrosis stage F3 is taken up to 14 kPa, and fibrosis stage F4, i.e. cirrhosis over 14 kPa. For the optimal cut-off for significant fibrosis, we take 9.9 kPa, and at a liver density below 7.9 kPa no evidence of advanced fibrosis.

When determining the degree of steatosis according to CAP, based on literature data from studies around the world and in Bulgaria, we accept the following cut-off values: S0 – for values below 215 dB/m, S1 – for values up to 253 dB/m, S2 – for values up to 300 dB/m and S3 for values above 300 dB/m.

Fig. 2 Fibroscan results of patients participating in the study

3.2.7. Statistical methods

The present work included various descriptive and analytical methods based on parametric and non-parametric tests addressing the research objectives.

3.2.7.1. Descriptive methods

Descriptive analysis was used to describe the main characteristics of the sample and the indicators included in the study. Central tendency measures such as arithmetic mean and non-parametric tests such as crosstabulation and hi-square were used as the basis of the

analysis in search of significant differences in frequency representation of categorical values (number and percentages). Statistical significance in statistical tests was accepted at $p \leq 0.05$.

3.2.7.2 Analytical methods

Independent T-test was used to compare the mean values of quantitative indicators of two groups for qualitative signs. Differences between groups were statistically significant at $p \leq 0.05$. Analysis of variance (ANOVA) was used to compare differences in the clinical picture of the patients. The graphical visualization of the compared values of the indicators are presented with Error Bar Graphs. The differences between the values were considered reliable at the $p<0.05$ value accepted for biological experiments. Multiple comparisons between groups were made with Tukey's post-hoc analysis. Differences between groups were statistically significant at $p \leq 0.05$.

3.2.7.3. Correlation analysis was used to examine the relationships between clinical parameters and to establish the strength of their influence. The assessment of the strength of the dependence between the variables is based on the results of the Pearson coefficient (r) - for quantitative indicators and of Spierman (rho) for quantitative and qualitative indicators. The degree of association between variables was defined as significant at $r > 0.5 < r=0.7$; large at $0.7< r =0.9$ and extremely large at $r > 0.9$ at $p \leq 0.05$.

3.2.7.4. Regression analysis (R) was used to examine functional relationships between quantified traits. Regression analysis was tested to establish the magnitude and weight of each of the variables in the selected models of variables explaining their predictability.

4. Results

4.1. Demographic data - distribution of the examined persons by gender and age

148 patients were included in the study. The mean age of the studied patients was 55.68±13.20 years, with a minimum age of 23 years and a maximum of 82 years. Of these, 65 (44%) were men and 83 (56%) were women (Fig. 3).

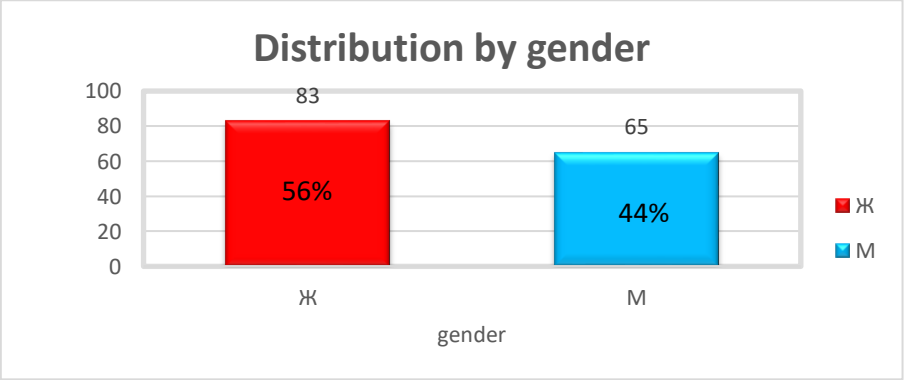


Fig. 3 Distribution by gender

The largest number (n=15) of men were from the age group 50-59 years, followed by the interval - 40-49 years (n=14) and the same number in the interval 60-69 years (n=14), 11 were in the interval - 30-39 years, 6 - in the age group 70-79 years, and 5 in the interval 20-29 years. There were no men in the age group over 80 years. The largest number of women (n=29) were from the 50-59 age group, followed by 24 women from the 60-69 age group, 17 women in the 70-79 age range, 6 women in the 40 age range -49 years, 5 – in the interval 30-39 years, 1 – from 20 to 29 years and 1 patient over 80 years old (Fig. 4 and Table 3).

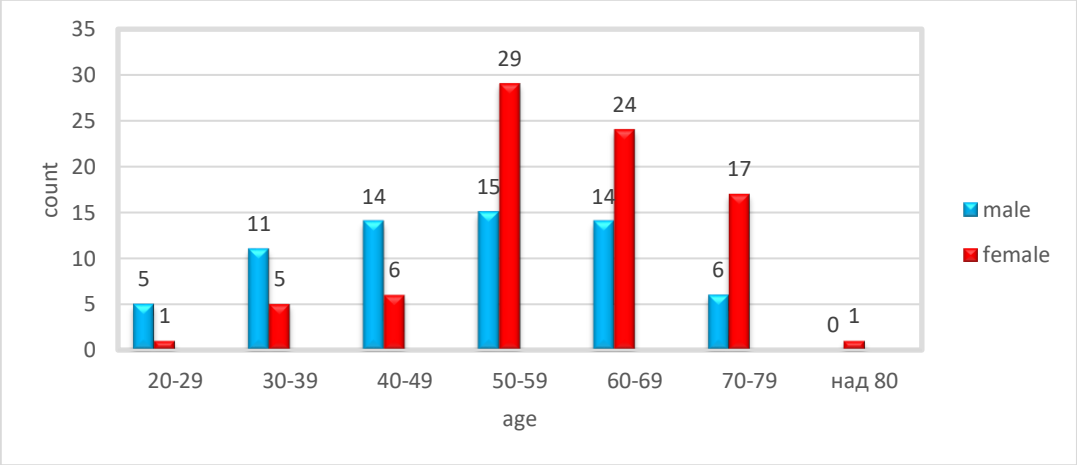


Fig. 4 Distribution by gender and age

age	Men	Women	total
20-29	5	1	6
30-39	11	5	16
40-49	14	6	20
50-59	15	29	44
60-69	14	24	38
70-79	6	17	23
Above 80	0	1	1
Total	65	83	148
Average age (years)	50,44	59,78	55,68
Standard deviation (years)	13,95	11,03	13,202
Minimum age	23	28	23
Maximum age	77	82	82

Table 3 Distribution by gender and age

4.2. Clinical characteristics of patients

After excluding comorbidities that may lead to steatosis and after excluding significant ethanol intake, it is particularly important for the clinical evaluation of patients with NAFLD to specify the presence of components of the metabolic syndrome.

4.2.1. Type 2 DM in the study group of patients with NAFLD

In our studied group of 148 individuals, proven DM type 2 was present in 70 patients (47.29%), of whom 44 were women and 26 men. Accordingly, there were 78 patients without

known DM type 2, but in 23 of them, an elevated fasting glycemia was found with serum glucose level between 5.6 mmol/l and 6.9 mmol/l, which formed the prediabetes group (15.55%), including 10 men and 13 women. There were 55 (37.16%) persons without proven type 2 DM and no evidence of prediabetes, of whom 26 were women and 29 were men (Fig. 5 and Ta ble 4).

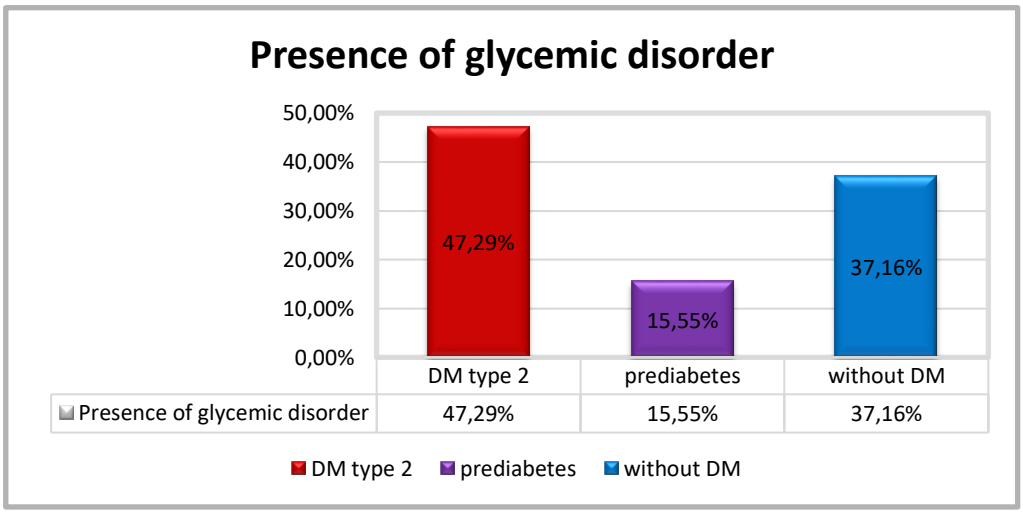


Fig. 5 DM type 2, prediabetes and absence of diabetes

	Men	Women	Total	Percentage
DM type 2	26	44	70	47,29%
prediabetes	10	13	23	15,55%
Absence of DM	29	26	55	37,16%

Table 4 Distribution by gender and presence of type 2 DM, prediabetes and without type 2 DM

4.2.2. Hypertensive Heart Disease

The majority of patients had a history of proven hypertensive disease undergoing antihypertensive treatment (n=120 of a total of 148; 81.08%), of which 46 were men and 74 were women (Fig. 6). There were 28 patients (18.92%, men - 19, women - 9) without a history of proven hypertensive disease and without physical evidence of increased arterial blood pressure (Fig. 5)

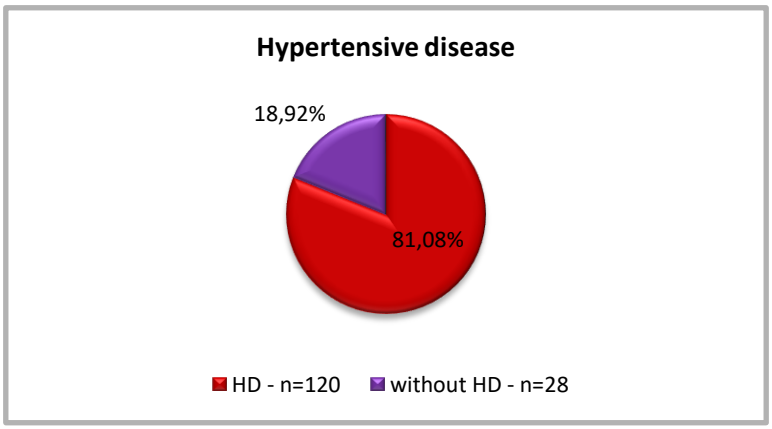


Fig. 5 Distribution of patients according to the presence of HD

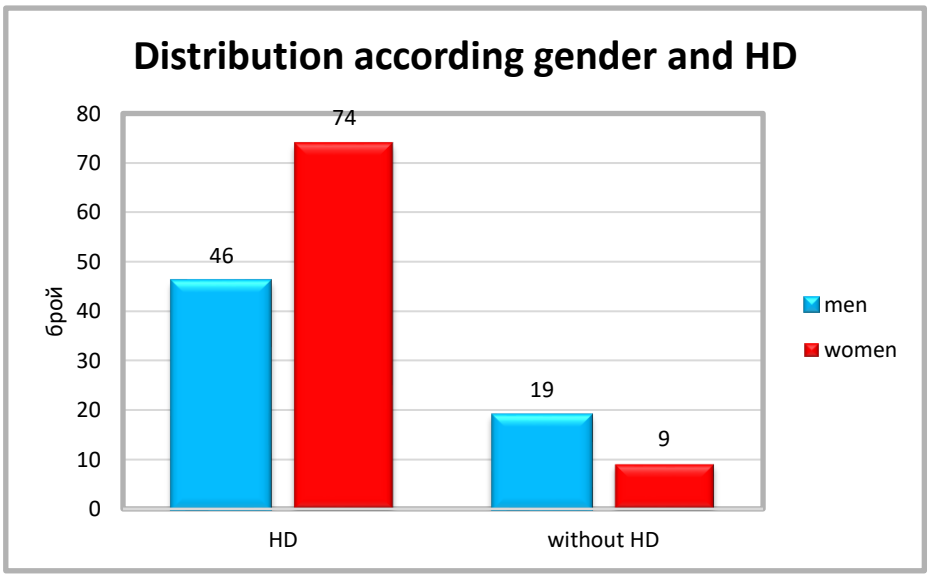


Fig. 6 Distribution of patients by gender according to the presence of HD

The analysis of the distribution of patients according to the stage of the hypertensive heart disease showed that out of a total of 120 patients with HD, with stage I there were 5 patients with an average age of 51.2±11.07 years, with stage II HD there were 78 patients with an average age of 55 ,61±11.10 years, with III stage HD there are 37 patients with an average age of 67.02±7.51 years (table 7).

HD stage	Patients	Percentage	Average age
HD I st.	5	4,17%	51,2±11,07
HD II st.	78	65,00%	55,61±11,10
HD III st.	37	30,83%	67,02±7,51

Table 7 Distribution according to stage of HD

4.2.3. Results for body weight and height.

The average height found in men was 1.75±0.08 m, and in women it was 1.62±0.06 m. The measured average body weight was 94.18±20.42 kg, minimum 43kg and maximum 163kg. In men, the average body weight was 102.71±20.12 kg., minimum 67.3 kg. and a maximum of 145 kg. For women, the determined average weight was 87.80±18.32 kg., minimum 43 kg. and a maximum of 163 kg.

4.2.4. Obesity grade and BMI results

Body mass index was calculated in 131 patients, the mean value is 33.47±6.74. The values were very similar for both sexes, 33.40±6.05 in males (n=56) and 33.52±7.25 in females (n=75), respectively.

7 out of 131 patients (5.34%, 3 men and 4 women) had a normal body weight (BMI 18.5-24.9). 33 patients (25.19%, 12 men and 21 women) were overweight (BMI 25-29.9). There were 48 patients (36.64%, 22 men and 26 women) with grade I obesity (BMI 30-34.9). There were 24 patients (18.33%, 11 men and 13 women) with grade II obesity (BMI 35-39.9). There were 19 patients (14.50%, 8 men and 11 women) with grade III obesity (BMI ≥40). With

the highest BMI - 66.98 is a 39-year-old woman with a body weight of 163 kg. and height 1.56 m. The results are summarized in Table 8, Figure 6 and 7.

	Men – 56	Women-брой -75	Total	Percentage
Normal weight	3	4	7	5,34%
Overweight	12	21	33	25,19%
Obesity I st.	22	26	48	36,64%
Obesity II st.	11	13	24	18,33%
Obesity III st.	8	11	19	14,50%

Table 8 Distribution by gender and BMI

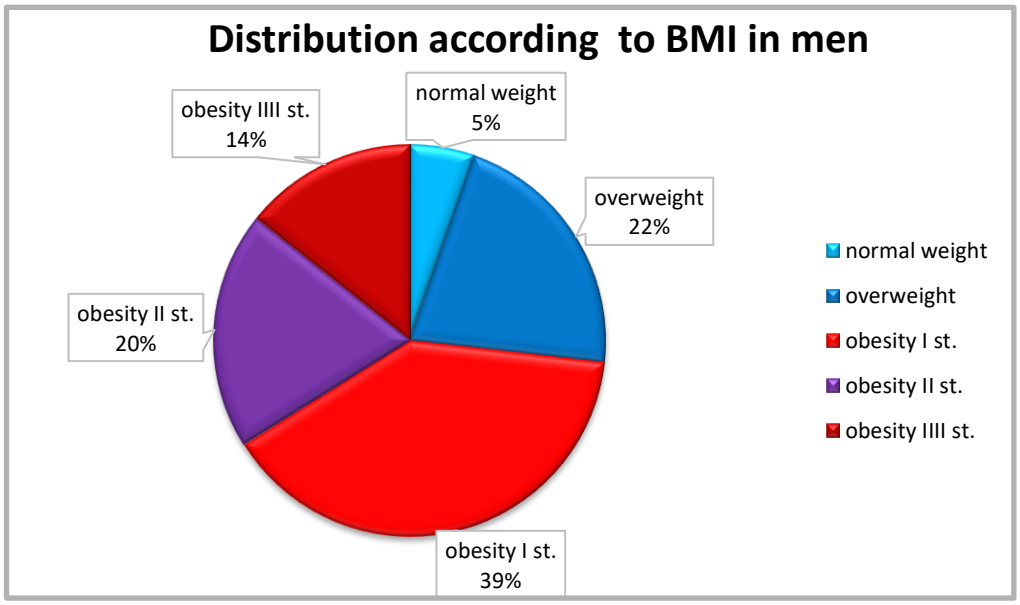


Fig. 7 Distribution according to BMI in men

Fig. 7 Distribution according to BMI in women

4.2.5. Waist circumference results

Abdominal circumference is one of the most important anthropometric indicators for the presence of visceral obesity, which is a sign of the metabolic syndrome and a prerequisite

for the appearance of hepatic steatosis. Therefore, determination of waist circumference is important in the clinical evaluation of patients with NAFLD.

In the group studied by us, the measured mean waist circumference was 113.21 ± 13.41 cm. in a total of 126 patients (52 men and 74 women), a minimum value of 85 cm. and a maximum of 150 cm. (fig. 8). In all 74 women, the waist circumference was over 80 cm. and in all 52 men the waist circumference was above 94 cm., which limit values according to the WHO for the European race determine the presence of visceral obesity and are associated with a higher health risk. In women, the average waist circumference was 111.12 ± 13.10 cm. (85-146 cm.), and in men 116.19±13.41 (94-150 cm.).

According to the WHO, two levels of visceral obesity are defined, associated with increased and significantly increased risk for metabolic diseases and health risk (Table 12). In the group studied by us, according to these criteria, waist circumference in men ≥ 102 cm had 46 patients (88.46%), the remaining 6 patients (11.53%) had waist circumference ≥ 94 cm and < 102 cm. Among women, 73 (98.65%) had waist circumference ≥ 88 cm and only 1 (1.35%) had 2waist circumference ≥ 80 cm and < 88 cm.

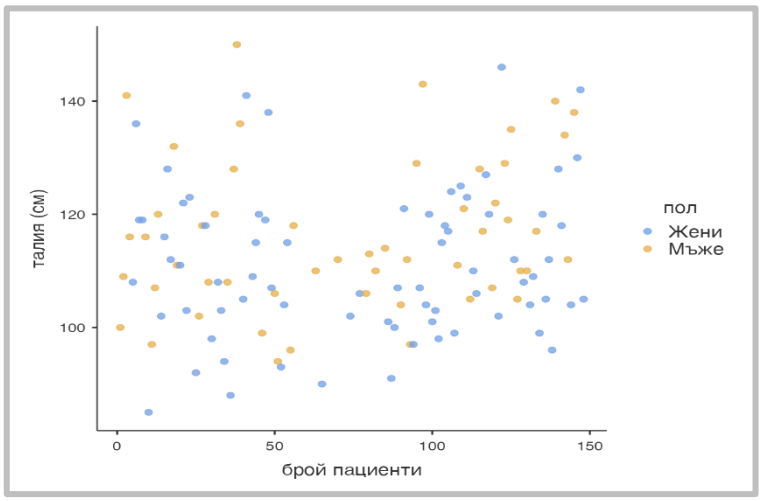


Fig. 8 Distribution according to abdominal circumference in men and women.

4.2.6. Metabolic syndrome and HOMA-IR

The metabolic syndrome, as a complex of risk factors, underlies NAFLD, which is its hepatic phenotypic manifestation. In our study group of 148 individuals, 131 had more than three criteria for MS, therefore its presence was accepted (55 men and 76 women). In the remaining 17 patients, only one or two criteria for MS were found (10 men and 7 women). The results are presented in table 9 and figure 9.

Gender	Metabolic syndrom		Total
	Yes (≥ 3 criteria)	No (1 or 2 criteria)	
Women	76	7	83
Men	55	10	65
Total	131	17	148

Tabl. 9 Presence of metabolic syndrome

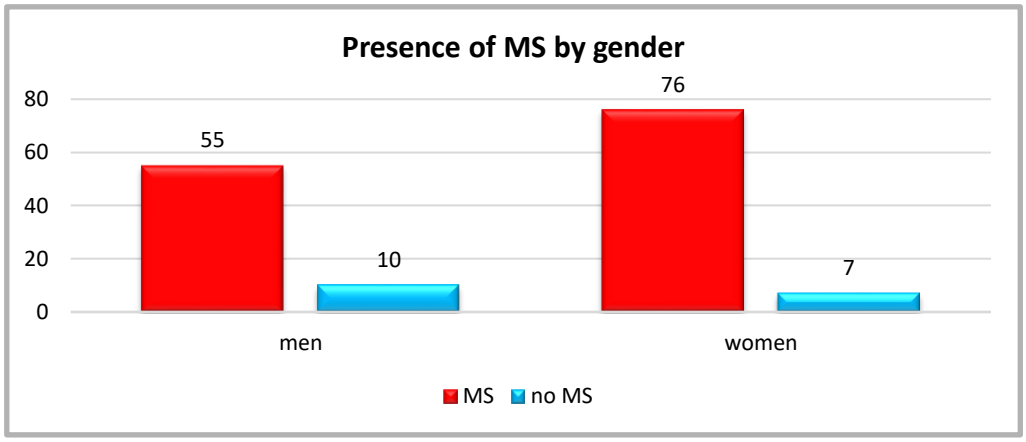


Fig. 9 Presence of MS by gender

HOMA-IR was calculated in 61 patients. The average value found was 6.15±8.43, minimum value 0.77 and maximum 56.84. Values greater than 2.5 were found in 44 patients and less than 2.5 in 17 patients. Values above 5.0 were found in 24 patients (Fig. 10).

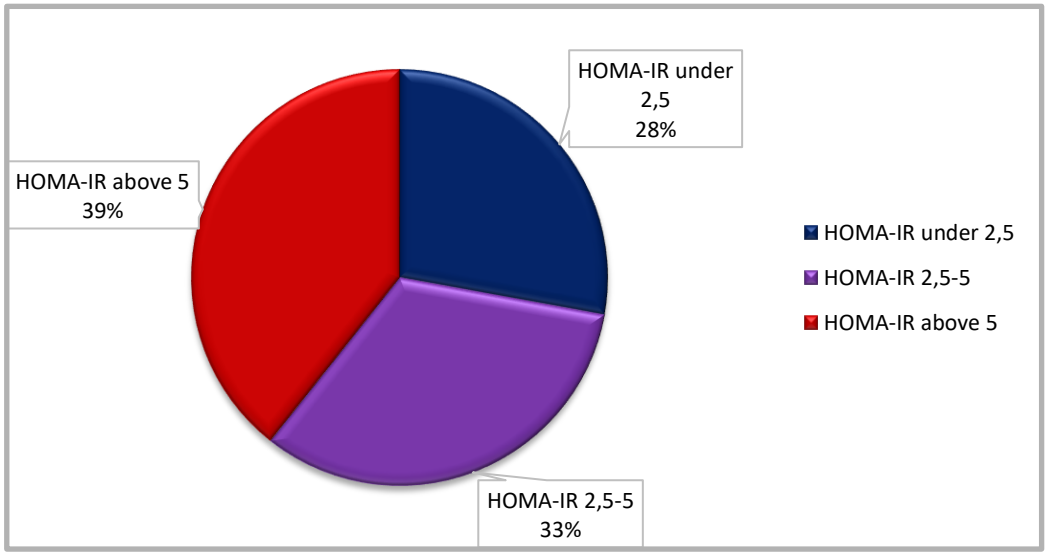


Fig. 10 Distribution by value of HOMA-IR

4.2.7. Laboratory test results relevant to the clinical characteristics of patients with NAFLD

4.2.7.1. Values of ALT, AST, GGT, AF and total bilirubin

The calculated mean value of ALT was 41.73±50.28, and above the upper reference limit of 49 U/l was found in 35 patients (in total n=147), i.e. in 23.80% there is an increase in ALT.

The calculated mean value of AST was 33.15±27.68, and above the upper reference limit of 34 U/l was also found in 35 patients (in total n=147), i.e. in 23.80% there is an increase in ASAT.

GGT was studied in 109 patients (45 men and 64 women), and its mean value was 64.94±88.14 in men and 49.08±55.61 in women, being above the upper reference limit (73 U/l

in men and 38 U/l in women) was found in 48 patients (25 men and 23 women), i.e. in total, an increase in GGT was observed in 44% of the examined patients.

AF was studied in 69 patients (43 men and 26 women), the mean value was 91.78±47.20. Values above the upper reference limit (129 U/l) were found in 7 patients – 10.14%, with the increase being slightly up to 2 times the norm.

The established mean value of total bilirubin was 11.42±6.64 (n=73). Values above the upper reference limit were observed in 6 patients (8.21%) and in a very mild degree up to a maximum of 2 times the norm. The increase in total bilirubin is primarily due to indirect bilirubin and is therefore likely associated with an underlying Gilbert's syndrome or an extrahepatic cause. The percentage increase in biochemical exams is presented in figure 11.

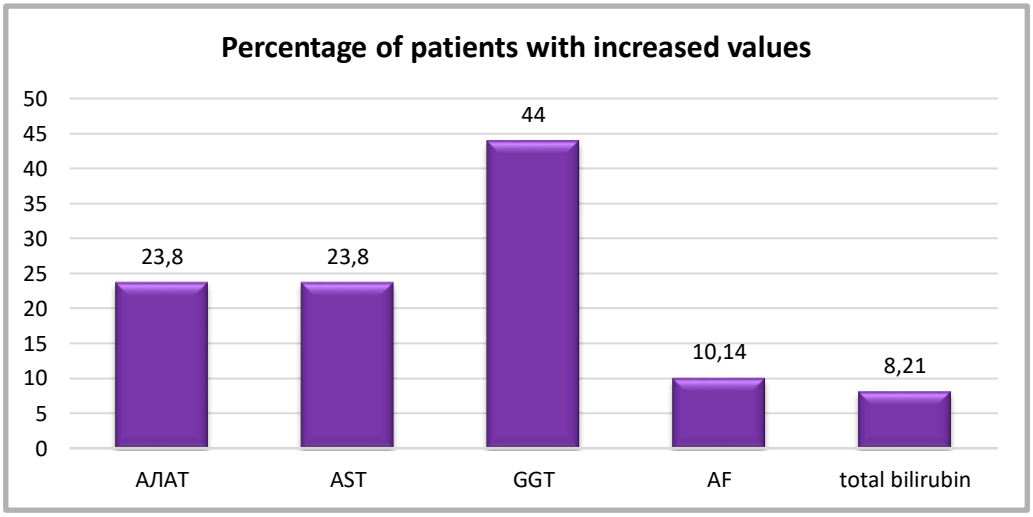


Fig. 11 Percentage of patients with increased values of AST, ALT, GGT, AF, total bilirubin

The highest percentage of patients was found to have an increase in GGT and the least with an increase in AF and bilirubin. Similar results from the analysis of laboratory tests in patients with hepatic steatosis were found in other Bulgarian studies (Table 10).

	ALT	AST	GGT	AF	Totl bilir.
Tomova 2021	52%	48%	85%	32,9%	3%
Balabanska 2021	45,3%	37,6%	50,6%	1,2%	/
Our data	23,8%	23,8%	44%	10,14%	8,21%

Table 10 Comparison with data from recent Bulgarian studies

Scientists from the American College of Gastroenterology, Stanford proposed in 2017 the adoption of new lower values of the lower limit of the norm for ALT and AST. In this way, underlying hepatocellular damage is established with greater certainty. It is known that normal values of liver enzymes do not rule out liver damage. Aminotransferase values depend on gender and age. ALT values of 29 to 33 IU/l for men and 19 to 25 IU/l for women are considered truly normal. Values above these are considered elevated. With the criteria proposed in this way, in the group of patients studied by us, with ALT over 33 IU/l were 39% of men (n=25, out of a total of 64 men), with ALT over 25 IU/l were 44.57% of women (n= 37 out of a total of 83 women). Accordingly, out of all 147 patients with ALT examined, there

were 62 patients - 42.17%, compared to only 23.8% with an elevated value according to the generally accepted u.l.n.

4.2.7.2. Results for triglycerides, HDL cholesterol, total cholesterol

The mean value of serum triglycerides in the studied patients (n=139) was 2.09±1.48 (from 0.58 mmol/l to 10.27 mmol/l). Elevated values (≥ 1.7 mmol/l) were observed in 66 patients (47.48%), of which 32 men and 34 women, respectively normal values were observed in 73 patients (52.52%), of which 28 men and 45 women (Fig. 12).

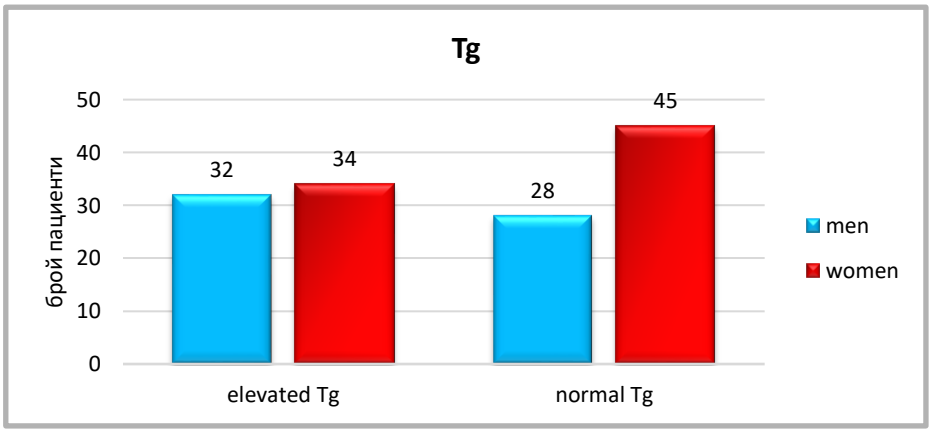


Fig. 12 Distribution of patients by gender and value of 3-GL

HDL cholesterol was measured in 67 patients, 30 men and 37 women. The mean serum value was 1.14±0.39 mmol/l (range 0.57 mmol/l to 1.47 mmol/l). Decreased values below 1.3 mmol/l in women were found in n=25, and higher values in 12 patients. In men with decreased values below 1.0 mmol/l there are 15 and the same number n=15 with normal values. In total, there were 40 (59.7%) patients with low HDL cholesterol and 27 (40.3%) with normal (Fig. 13).

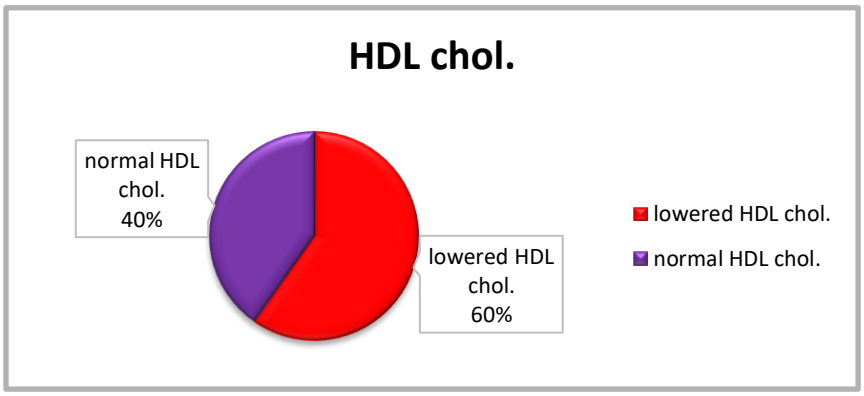


Fig. 13 Distribution by changes in HDL cholesterol value

The level of total serum cholesterol was measured in 118 patients, 51 men and 67 women (from 2.55 mmol/l to 7.95 mmol/l). The mean value was 5.24±1.2 mmol/l. Elevated values above 5.18 mmol/l were found in 66 patients, 56% (27 men and 39 women), and normal values in 52 patients, 44% (24 men and 28 women).

4.2.8. Summarized demographic, clinical and laboratory data for the study group of patients presented by gender

Descriptives – men					
	age	**BMI=**	**weight**	**waist (cm)**	**height**
N	65	56	56	52	56
Mean	50.4	33.4	103	116	1.75
Median	51	32.8	104	113	1.76
Standard deviation	14.0	6.05	20.1	13.4	0.0839
Minimum	23	22.0	67.3	94	1.50
Maximum	77	51.4	145	150	1.92

Descriptives -women					
	age	**BMI=**	**weight**	**waist (cm)**	**height**
N	83	75	75	74	76
Mean	59.8	33.5	87.8	111	1.62
Median	60	32.4	85.7	109	1.62
Standard deviation	11.0	7.25	18.3	13.1	0.0624
Minimum	28	20.2	43.0	85	1.46
Maximum	82	67.0	163	146	1.76

Descriptives – men								
	Hb	**Trb**	**CRP**	**Tg**	**HDL chol.**	**LDL chol.**	**chol.**	**CK 18**
N	50	63	30	60	30	30	51	23
Mean	150	251	12.2	2.35	1.31	3.01	5.27	4.70
Median	152	249	2.67	1.75	1.01	3.07	5.37	0.900
Standard deviation	15.5	61.1	27.9	1.65	1.60	1.10	1.22	16.5
Minimum	112	132	0.0800	0.860	0.570	0.820	1.87	0.100
Maximum	191	400	143	9.10	9.70	5.80	7.95	80.0

Descriptives - women								
	Hb	**Trb**	**CRP**	**Tg**	**HDL chol.**	**LDL chol.**	**chol.**	**CK 18**
N	69	83	45	79	37	38	67	38
Mean	130	283	7.79	1.91	1.24	2.94	5.22	1.99
Median	132	270	3.56	1.59	1.10	2.98	5.39	0.800
Standard deviation	18.1	79.7	19.9	1.32	0.476	1.03	1.20	3.50
Minimum	66	151	0.100	0.580	0.760	1.29	2.84	0.100
Maximum	164	550	135	10.3	2.91	6.23	8.20	16.0

Descriptives – men						
	AST	**ALT**	**GGT**	**Total bilir.**	**Alb.**	**AF**
N	64	64	45	26	62	26
Mean	31.2	43.7	64.9	15.2	44.6	88.5
Median	26.0	29.0	40.0	14.2	44.0	75.0
Standard deviation	24.7	58.3	88.1	8.19	4.32	39.9
Minimum	6.50	7.50	12.0	6.00	33.0	48
Maximum	172	448	515	42.0	57.0	211

Descriptives- women						
	AST	**ALT**	**GGT**	**total bilir.**	**alb.**	**AF**
N	83	83	64	47	83	43
Mean	34.6	40.2	49.1	9.33	43.5	93.8
Median	23.0	22.0	27.5	9.00	43.8	81
Standard deviation	29.8	43.4	55.6	4.47	3.40	51.5
Minimum	11.0	5.00	8.00	2.50	36.0	54
Maximum	178	283	354	23.0	50.1	329

Table 11 Summarized demographic, clinical and laboratory data presented by gender

Laboratory data (n-count)	median	SD	Normal (n, %)	Abnormal (n, %)
ALT (147)	41,73	50,28	112 (76,2%)	Eleveted-35 (23,8%)
AST (147)	33,76	27,68	112 (76,25%)	Elevated -35 (23,8%)
GGT(109)	64,94-men 49,08-women	88,14 55,61	61 (56%)	Elevated -48 (44%)
AF (69)	91,78	47,20	62 (89,86%)	Elevated -7 (10,14%)
Total bilir. (73)	11,42	6,64	67 (91,79%)	Elevated -6 (8,21%)
Trb. (146)	269,36	73,82	144 (98,6%)	Lowered -2 (1,4%)
Tg (139)	2,09	1,48	73 (52,52%)	Elevated - 66 (47,48%)
HDL chol. (67)	1,14	0,39	27 (40,35%)	Lowered - 40 (59,7%)
total chol. (118)	5,24	1,2	52 (44%)	Elevated -66 (56%)
albumin (147)	43,98	3,83	147 (100%)	0%

Table 12 Summary of abnormal laboratory tests

4.2.9. Abdominal ultrasound

Abdominal ultrasound was performed in all 148 patients. The echogenicity of the liver, relative to the parenchyma of the right kidney, the visualization of the intrahepatic vessels, the diaphragm, the posterior segments of the right lobe and the wall of the gallbladder was evaluated, based on which the steatosis was graded as mild, moderate and severe. In 3 patients with metabolic syndrome, there was no ultrasound evidence of steatosis. In the subsequent fibroscan with measured CAP, data on mild steatosis were found in two of these patients, and in one the values were borderline compared to our accepted cut-off for the presence of steatosis (211 dB/m versus 213 dB/m). In 28 patients, steatosis was assessed as mild (1 percent), of which 14 were men (50%) and 14 (50%) were women. In 51 patients, steatosis was assessed as moderate (2 percent), of which 19 (37.25%) were men and 32 (62.75%) were women. In 66 patients, steatosis was assessed as pronounced (3 points), of which 31 (46.97%) were men and 35 were women (53.03%). Accordingly, of all 148 patients, 2% were evaluated sonographically without steatosis (n=3), 19% with mild steatosis (n=28), 34.4% were evaluated with moderate steatosis (n=51) and 44.6% with severe steatosis (n=66) (Figs. 14 and 15).

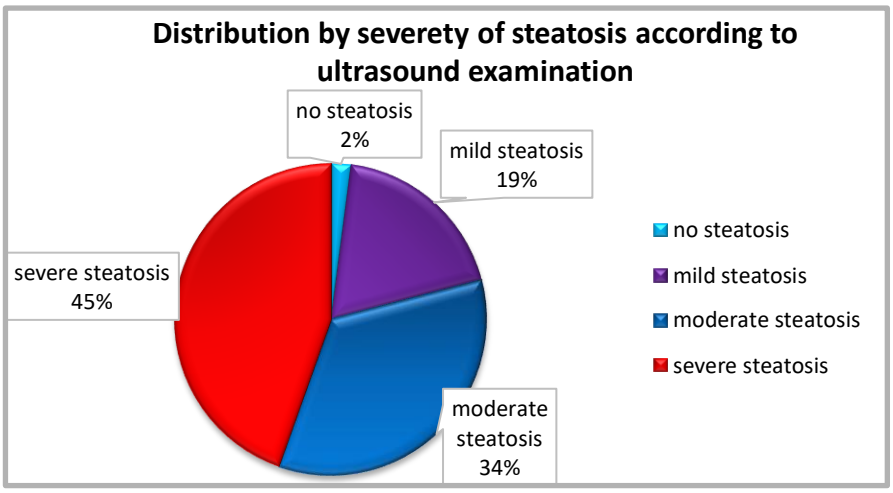


Fig. 14 Distribution by severity of steatosis according to ultrasound examination

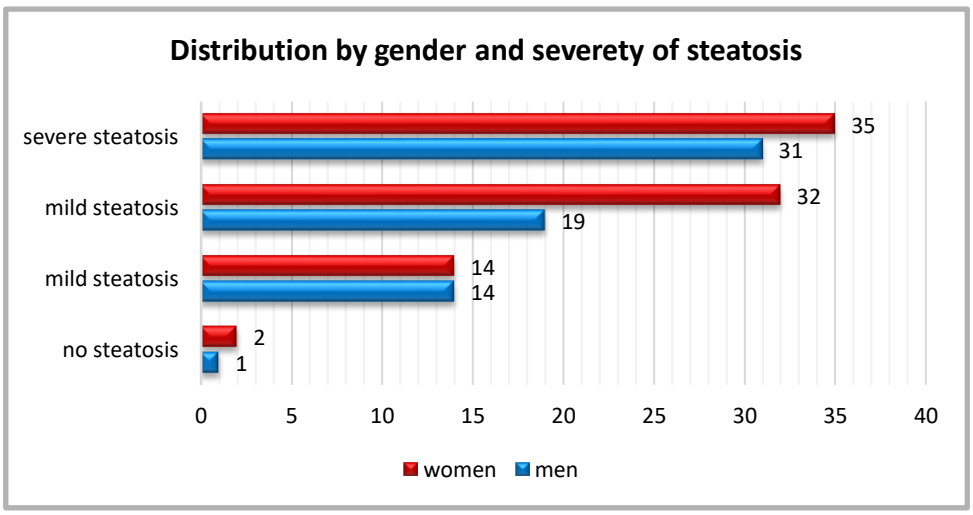


Fig. 15 Distribution by gender and severity of steatosis according to ultrasound examination

Liver anteroposterior size along the right medioclavicular line (RMCL) was also measured. The accepted normal value for maximum liver size is 140 cm. In 104 patients, hepatomegaly with liver size over 140 mm was found. The maximum measured value was 224 mm., in two 52-year-old men with marked steatosis.

The presence of a focal sparing was assessed, and in 34 patients it was found in a typical place around the gallbladder in the right lobe. The surface and structure of the liver are described, its elasticity and the presence of data on portal hypertension are assessed.

In 2 of the patients, echographic evidence of liver cirrhosis was established, which was confirmed by the fibroscan performed afterwards with measurement of liver density. The patients are women aged 55 and 73, with evidence of metabolic syndrome. Accordingly, the F values in these patients were 24.5 kPa and 20.9 kPa. The patients have a normal synthetic liver function and during the performed gastroscopy they have no esophageal varices.

In 8 patients out of all 148, given the descriptive and subjective assessment of the ultrasound examination, reduced liver elasticity was found (based on the transmission of heart

beats on the right lobe of liver and change of the angle of the left lobe during inspiration). 4 of these patients were from the group with a fibroscan, and one of them had significant fibrosis F - with a value of 10.3 kPa, 1 had a borderline value of absent/advanced fibrosis of 7.9 kPa, and the remaining 2 had a normal liver density 6.5 kPa and 3.3 kPa respectively. In the remaining 4 patients, the determined fibrosis scores were below the values defining the presence of significant fibrosis.

4.2.10. Results of assessing the degree of steatosis with CAP

Assessing of the ultrasound attenuation parameter (CAP, controlled attenuation parameter) with fibroscan was performed in 84 patients. The measured average value of CAP is 304.39±47.50 dB/m, with a minimum value of 211 dB/m and a maximum value of 400 dB/m (Fig. 16). The mean value in men (n=43) was 304.33±46.85 dB/m, with a minimum value of 211 dB/m and a maximum value of 400 dB/m. In women (n=41), the mean value was similar 304.46±48.75 dB/m, with a minimum value of 219 dB/m and a maximum value of 400 dB/m (Fig. 17).

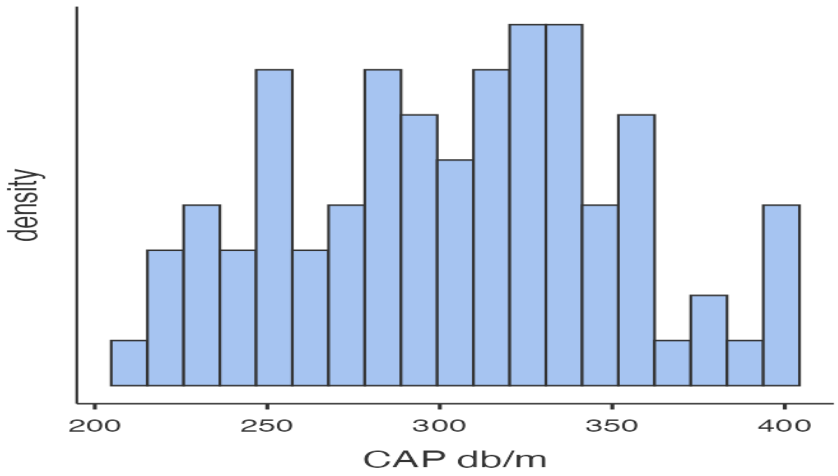
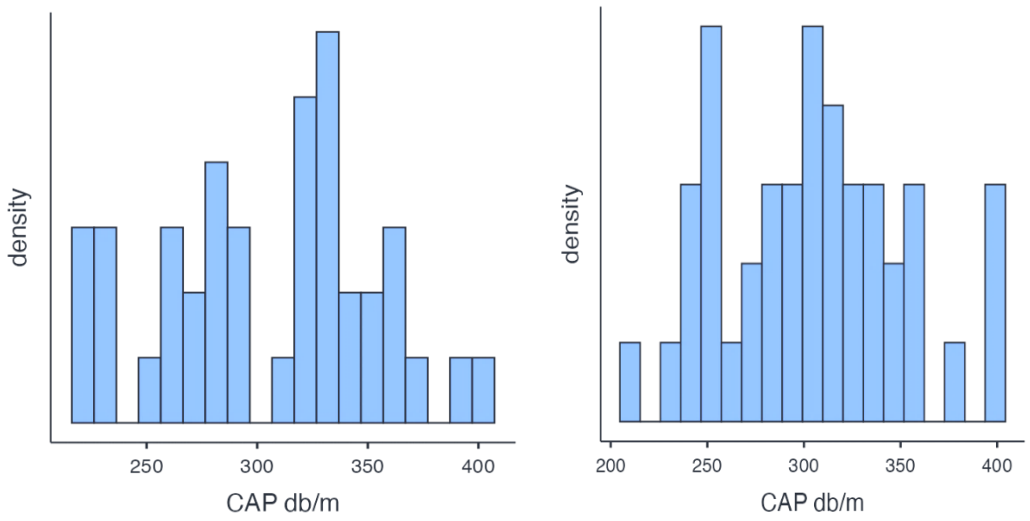


Fig. 16 Measured CAP values in dB/m in the studied group



men women

Fig. 17 Measured CAP values in dB/m for men and women

When assessing the degree of steatosis according to CAP, based on literature data from studies and meta-analyses around the world and in Bulgaria, we accepted the following cut-off values: S0 – for values below 215 dB/m, S1 – for values up to 253 dB/m, S2 – for values up to 300 dB/m and S3 for values above 300 dB/m.

According to accepted data, respectively, only 1 patient has grade S0, without steatosis - 1.19%, with grade S1 are 13% of patients, n=11, grade S2 are 27 patients, respectively 32.14% of the total number, are grade S3 - 45 patients, respectively 53.58% of the total number examined (Fig. 18).

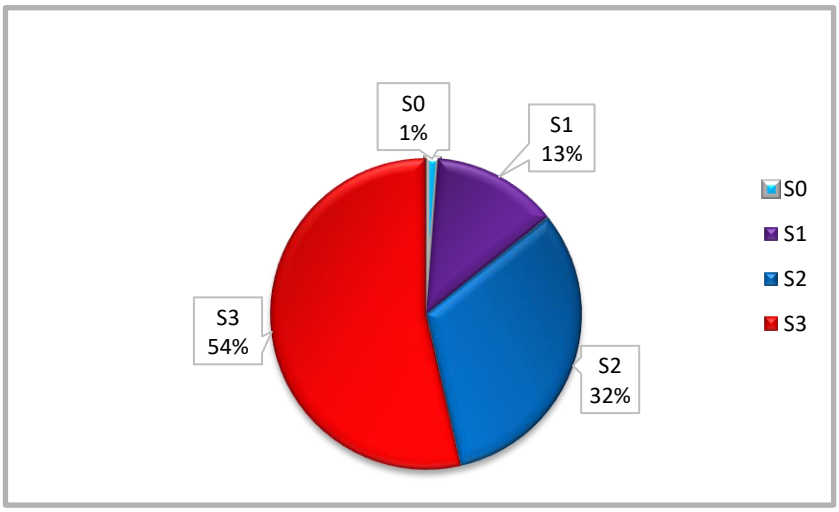


Fig. 18 Distribution by severity of steatosis according to CAP

The distribution by gender and degree of steatosis was respectively: S0 – 1 man, S1 – 11 patients, of which 5 men (45.45%) and 6 women (54.55%), S2 – 27 patients, of which 14 men (51.85%) and 13 women (48.15%), S3 – 45 patients, of which 23 men (51.11%) and 22 women (48.89%) (Fig. 18). The gender distribution of the different degrees of steatosis is almost the same.

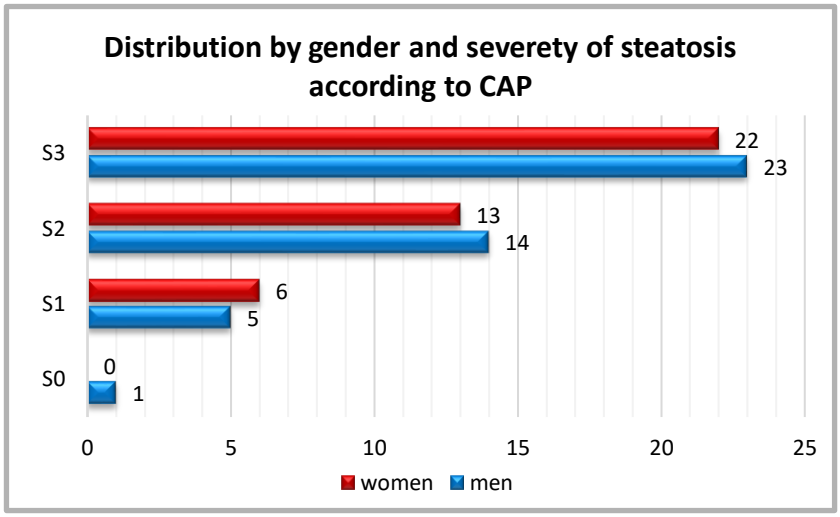


Fig. 18 Distribution by gender and severity of steatosis according to CAP

The distribution according to the degree of steatosis in men (n=43) was respectively S0 – 1 patient 2%, S1 – 5 patients 11%, S2 – 14 patients 33%, S3 – 23 patients 54%. In women (n=41) – S1 - 6 patients 15%, S2 – 13 patients 32%, S3 – 22 patients 53%.

Among the examined group of 84 patients with ultrasound and CAP, steatosis was staged by ultrasound, as: S0, without steatosis - n=3 (3.5%), S1 (mild) - n=17 (20.23%), S2 (moderate) – n=28 (33.37%) and S3 (severe) – n=36 (42.9%). A comparison of the data from ultrasound and CAP in these patients is presented in Fig. 20.

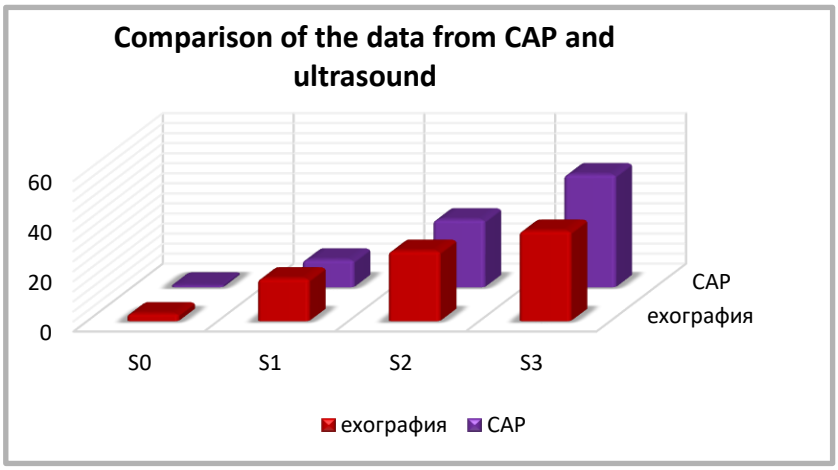


Fig. 20 Comparison of the data from CAP and ultrasound for distribution by degree of steatosis

4.2.11. Results of the studied scores for steatosis

- *HSI – Hepatic steatosis index* – score including ALT, AST, BMI, gender, presence of DM. Values <30 were considered to rule out NAFLD, and values >36, HSI was considered to be NAFLD. It was calculated in 130 patients, with a mean value being 45.79±7.69, minimum value 27.43, maximum 81.92. Only one patient had an index value below 30 - 27.43, and 125 patients had a value above 36. The remaining 4 patients were in the gray area between 30 and 36. The HSI is a simple and efficient screening tool for NAFLD that can to be used to select individuals for liver ultrasound and to determine the need for lifestyle modification.

- *LAP – Lipid Accumulation Product* – score including waist circumference, Tg, gender. The suggested values for validating the test are: LAP value <20 - no steatosis, while liver steatosis is accepted for LAP ≥80. LAP was accecced in 122 patients, with an average value of 110.30±84.33, minimum 27.60, maximum 587.59. Accordingly, the studied group lacks a patient with a value below 20, and 70 patients were with a value above 80. There were 52 patients with an intermediate value between 20 and 80.

- *Fatty liver index (FLI)* – score including BMI, waist circumference, Tg level and GGT. Index values range from 0 to 100. FLI below 30 is considered to rule out steatosis, and FLI greater than or equal to 60 is considered to prove steatosis. FLI was determined in 88 patients, with a mean value of 19.87±23.67, a minimum value of 0.9 and a maximum value of 98.08. An FLI value of less than 30 was found in 71 patients, and a value of more than 60 was found in only 7 patients. There are 10 patients with an intermediate value between 30 and 60.

- *NAFLD liver fat score* – score including presence of DM, serum insulin, AST, AST/ALT ratio. A value > -0.64 with a sensitivity of 86% and a specificity of 71% is considered as the optimal cut-off value. It was calculated in 61 patients, with a mean value of 1.8±3.85, a minimum value of -4.82 and a maximum value of 22.86. A score above -0.64 was found in 52 patients, and a score below -0.64 was found in 9 patients.

4.2.12. Statistical analysis of the results of measurement of steatosis by fibroscan CAP (Controlled Attenuation Parameter) and its relation with the clinical characteristics of the patients, defined scores for steatosis and comparison with results of fibrosis by fibroscan.

Correlation analysis was used to examine the relation between clinical parameters and establish the strength of their influence on the degree of steatosis measured by CAP. The assessment of the strength of the dependence between the variables is based on the results of the Pearson (r) and Spierman (rho) coefficients.

The results of the correlation analysis showed a strong degree of association between BMI and degree of steatosis by CAP (r=0.503, p=0.001) testifying to the parallel increase in the degree of steatosis with increasing values of body mass index. A strong correlation was also observed between the CAP value and waist circumference (r=0.481, p=0.001). Correlation analysis showed a weak association between the CAP value and the degree of increase in triglycerides (r=0.010, p=0.929) (Table 13 and Fig. 21).

Correlation Matrix											
			CAP db/m		**BMI=**		**waist (cm)**		**Tg**		
CAP db/m	Pearson's r		—								
	p-value		—								
BMI=	Pearson's r		0.503	***	—						
	p-value		< .001		—						
waist (cm)	Pearson's r		0.481	***	0.835	***	—				
	p-value		< .001		< .001		—				
Tg	Pearson's r		0.010		-0.029		0.022		—		
	p-value		0.929		0.748		0.810		—		
Note. * p < .05, ** p < .01, *** p < .001											

Table 13 Pearson correlation analysis of CAP versus BMI, abdominal circumference and triglycerides

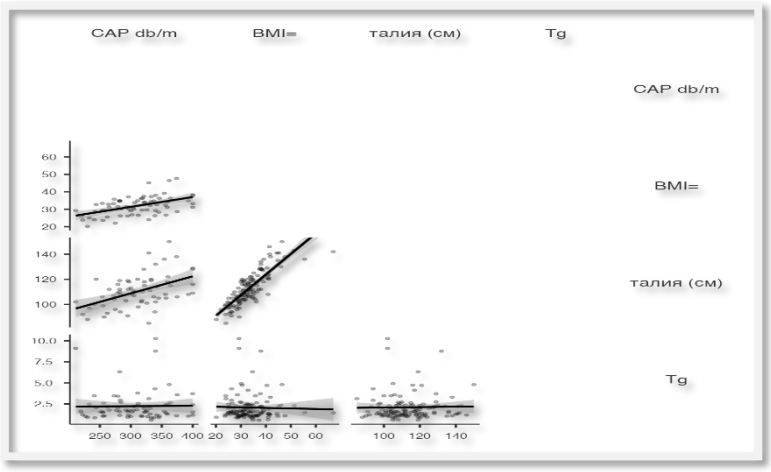


Fig. 21 Graphic representation of the above results

Correlation analysis according to the Spearman method was used to study the relation between the categorical clinical indicators - stage of HD, presence of DM and metabolic syndrome to establish the strength of their influence on the degree of steatosis measured by CAP (table 14 and fig. 22). The analysis showed that there is a moderate positive relation between HD and the degree of steatosis (rho=0.277, p=0.011), therefore, the degree of steatosis increases in parallel with the increase in the degree of HD and its duration. There is also a statistically significant relationship between the presence of MS and DM, but the analysis of the results showed that in our study group the correlation dependence for these clinical indicators is weak, as the Spearman correlation coefficient is with a value of rho=0.203, p=0.064 respectively for MS and rho=0.209, p=0.056 for type 2 DM.

Correlation Matrix											
				CAP db/m		**DM**		**MS**		**HD**	
CAP db/m		Spearman's rho		—							
		p-value		—							
DM		Spearman's rho		0.209		—					
		p-value		0.056		—					
MS		Spearman's rho		0.203		0.308	***	—			
		p-value		0.064		< .001		—			
HD		Spearman's rho		0.277	*	0.347	***	0.540	***	—	
		p-value		0.011		< .001		< .001		—	
Note. * p < .05, ** p < .01, *** p < .001											

Table 14 Correlation analysis by the Spearman method of CAP versus presence of type 2 DM, MS and stage of HD.

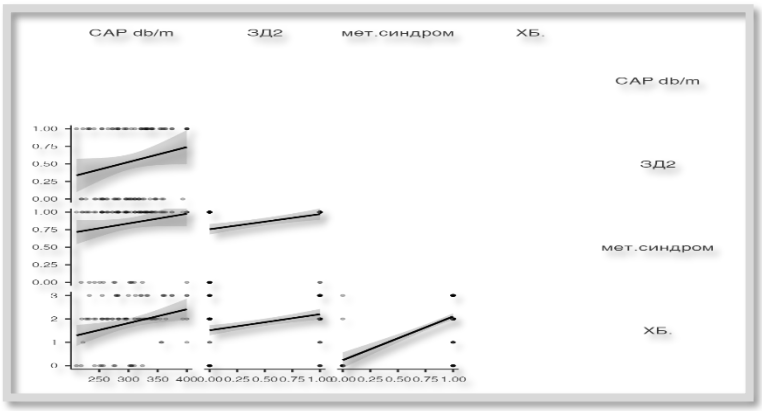


Fig. 22 Graphical representation of the above results

To establish the relationship between the components of the metabolic syndrome and CAP and to determine the quantitative relation between them and the degree of steatosis determined in CAP, i.e. in order to measure the factor influences and the specific effects of the influence of the MS components on the degree of steatosis, we used Regression Analysis (table 15). The components of MS - presence of HD, presence of glycemic disorders, increased Tg, increased waist circumference and changes in HDL - cholesterol are predictors for the appearance of hepatic steatosis and for determining its degree. All components are important, but the Regression model we compiled found that the increase in waist circumference, which is also the main factor presenting the degree of visceral obesity, has the greatest predictive power for the occurrence and degree of severity of steatosis determined by CAP (p= 0.002). The regression model showed that a 1.6 cm increase in waistline resulted in a reciprocal increase in the degree of steatosis by 2 dB/m.

Model Fit Measures

Model	R	R^2	Adjusted R^2
1	0.533	0.285	0.195

R=0.285, p<0.05

Model Coefficients - CAP db/m

Predictor	Estimate	SE	t	p	Stand. Estimate
Intercept	117.967	52.040	2.267	0.029	
DM	-7.289	15.864	-0.459	0.648	-0.0658
HD	6.376	8.094	0.788	0.435	0.1155
Tg	-0.761	3.343	-0.228	0.821	-0.0343
HDL chol.	0.853	5.323	0.160	0.874	0.0236
waist (cm)	1.683	0.495	3.396	0.002	0.4894

Table 15 Linear Regression - model for predictability of CAP dB/m from components of metabolic syndrome

Correlation analysis using the Spearman method was used to study the relation between the degree of steatosis determined by ultrasound and the steatosis determined by CAP. The results show a high degree of correlation (rho=0.462, p=0.001), indicating an increase in CAP with each degree of steatosis (table 16, fig. 23).

Correlation Matrix

		CAP db/m	US-grade of steatosis
CAP db/m	Spearman's rho	—	
	p-value	—	
US-grade of steatosis	Spearman's rho	0.462 ***	—
	p-value	< .001	—

Note. * p < .05, ** p < .01, *** p < .001, one-tailed

Table 16 Correlation between US grade of steatosis and CAP

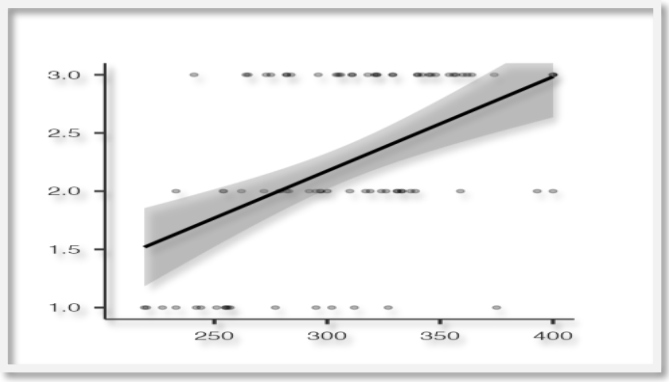


Fig. 23 Graphic representation of the correlation between the degree of steatosis determined sonographically and steatosis by CAP in dB/m

One-way ANOVA analysis was used to determine whether there were statistically significant differences between the three steatosis groups (Table 17, Fig. 24). A statistically significant difference was found between the three grades of steatosis (F=11.2, p=0.001).

One-Way ANOVA (Welch's)

	F	df1	df2	p
CAP db/m	11.2	2	41.1	< .001

Group Descriptives

	US-grade of steatosis	N	Mean	SD	SE
CAP db/m	1	17	267	42.3	10.25
	2	28	308	39.3	7.43
	3	36	326	39.9	6.66

Table 17 One-Way ANOVA analysis of the three grades of steatosis

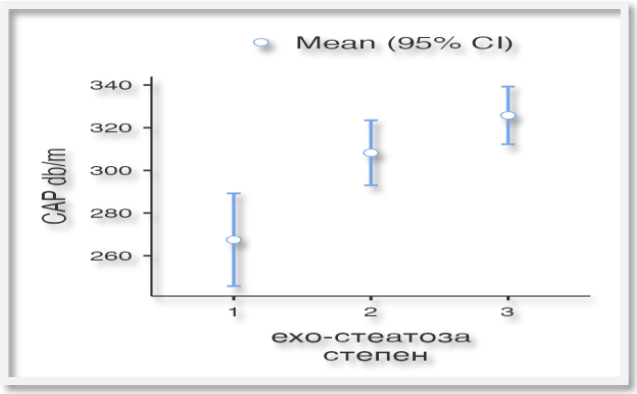


Fig. 24 Graphical representation of the above results

In order to specify exactly between which groups of steatosis there is a significant difference, we used the Tukey Post Hoc Test (Table 18). The mean values of the three grades of steatosis were compared. It was found that there is a significant difference in CAP values between first and second degree of sonographically determined steatosis (p=0.004) and

between 1st and 3rd degree (p=0.001), but no significant difference was found between 2nd and 3rd sonographically determined degree (p=0.201). Therefore, for a more accurate specification of the established ultrasound steatosis and the differentiation of moderate from severe steatosis, it is recommended to perform elastography with CAP.

Tukey Post-Hoc Test – CAP dB/m											
				1		**2**		**3**			
1		Mean difference		—		-40.8		-58.3			
		p-value		—		0.004		< .001			
2		Mean difference				—		-17.5			
		p-value				—		0.201			
3		Mean difference						—			
		p-value						—			

Table 18 Tukey Post Hoc Tests for the three degrees of steatosis

Correlation analysis according to the Pearson method was used to study the relations between CAP and the steatosis indexes - FLI (fatty liver index) and HSI (hepatic steatosis index) (table 19 and fig. 25). A moderately strong statistical relation was found for CAP and FLI (r=0.451, p=0.012), and for CAP and HSI (r=0.404, p=0.001), i.e. CAP increases in parallel with HSI & FLI.

Correlation Matrix											
				CAP db/m		**FLI**		**Hepatic steatosis index**			
CAP db/m		Pearson's r		—							
		p-value		—							
FLI		Pearson's r		0.451	*	—					
		p-value		0.012		—					
Hepatic steatosis index		Pearson's r		0.404	***	0.709	***	—			
		p-value		< .001		< .001		—			
Note. * p < .05, ** p < .01, *** p < .001											

Table 19 Pearson correlation analysis of CAP versus steatosis indexes - FLI and HSI

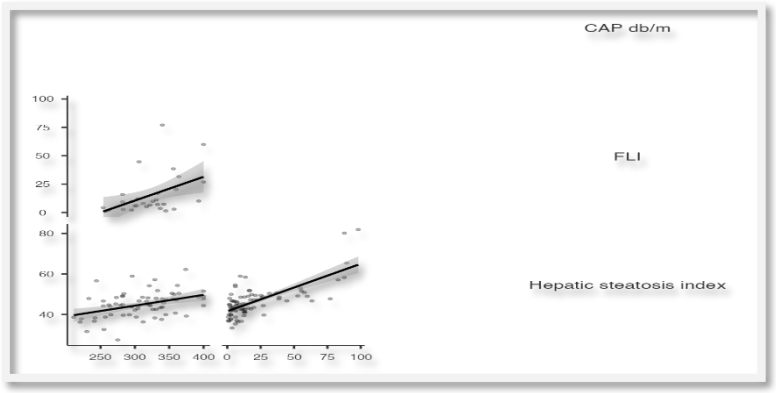


Fig. 25 Graphical presentation of the above results

One-way ANOVA analysis was performed to determine whether there were statistically significant differences between the three steatosis groups as determined by mean values of CAP and HSI and FLI (Table 20 and Fig. 26). Statistically significant differences were found between the three degrees of steatosis in the determined steatosis indexes, for HSI (F=6.55, p=0.003) and for FLI (F=11.86, p=0.001).

One-Way ANOVA (Welch's)

	F	df1	df2	p
Hepatic steatosis index	6.55	2	56.0	0.003
FLI	11.86	2	56.0	< .001

Group Descriptives

	ехо-стеатоза степен	N	Mean	SD	SE
Hepatic steatosis index	1	21	41.60	6.05	1.319
	2	45	45.43	4.81	0.717
	3	61	47.98	9.03	1.156
FLI	1	14	6.48	6.51	1.739
	2	36	14.65	15.96	2.660
	3	38	29.75	29.45	4.777

Tabl. 20 Comparison by groups of CAP steatosis and values of FLI and HSI

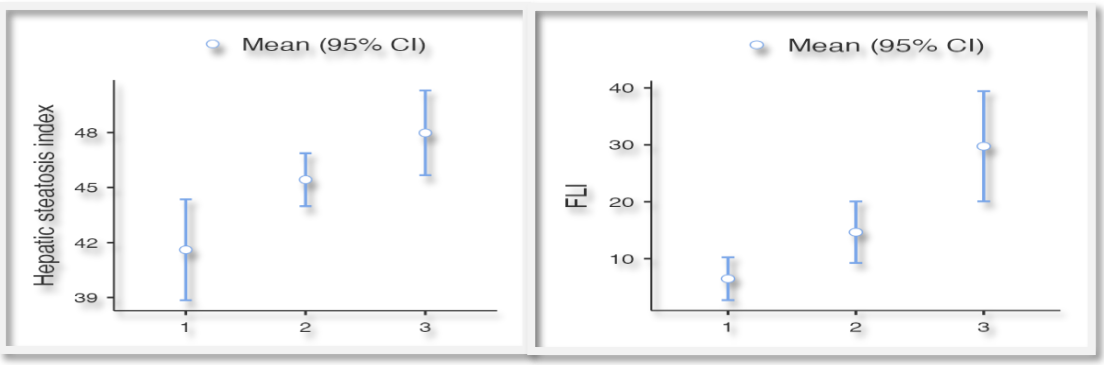


Fig. 26 Graphic presentation of the above data

We used Tukey's Post Hoc Test to specify exactly which steatosis groups had a significant difference in HSI and FLI values. The mean values for the three degrees of steatosis were compared. HSI was found to have a statistically significant difference between grade 1 and 3 steatosis (p=0.002), but no significant difference between grade 2 and 3 (p=0.182) and between grade 1 and 2 (p=0.122) (Table 21). For FLI, the data showed a sensitive difference between grades 1 and 3 (p=0.003), but also between grades 2 and 3 (p=0.012) (Table 22). Therefore, both indexes could be used as a screening for the presence of steatosis, and the FLI could better orient us to the degree of steatosis as well.

Tukey Post-Hoc Test – Hepatic steatosis index					
		1	2	3	
1	Mean difference	—	-3.83	-6.38	
	p-value	—	0.122	0.002	
2	Mean difference		—	-2.55	
	p-value		—	0.182	
3	Mean difference			—	
	p-value			—	

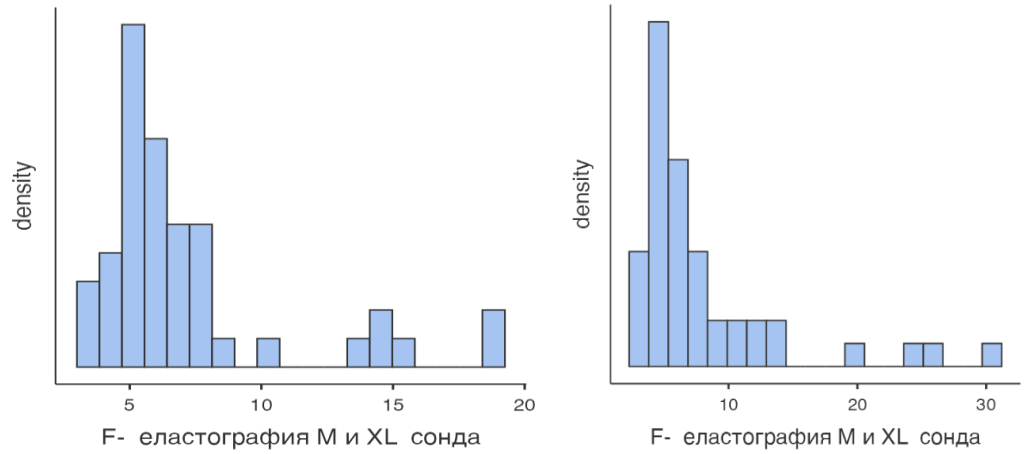
Table 21 Comparison between the three grades of steatosis to HSI

Tukey Post-Hoc Test – FLI					
		1	2	3	
1	Mean difference	—	-8.17	-23.3	
	p-value	—	0.472	0.003	
2	Mean difference		—	-15.1	
	p-value		—	0.012	
3	Mean difference			—	
	p-value			—	

Table 22 Comparison of the three grades of steatosis to FLI

4.2.13. Results of assessment of the stage of fibrosis by measuring liver density with fibroscan

Fibroscan liver density was measured in 91 patients and its mean value was 7.54±5.03 kPa, with a minimum value of 2.5 and a maximum of 29.9 kPa. In men (n=45) the mean value was 7.17±3.77 kPa, and in women (n=46) the mean value was 7.91±6.04 kPa (Fig. 27).



men women

Fig. 27 Measured values of liver density in kPa, by fibroscan in men and women

Assessment of fibrosis stage from 0 to 4 was performed according to accepted cut-offs from meta-analyses of referrals in patients with NAFLD and NASH. For normal elasticity we accept values up to 5-5.5 kPa, fibrosis stage F1 is accepted to 7 kPa, fibrosis stage F2 is accepted up to 10 kPa, fibrosis stage F3 is accepted up to 14 kPa, and fibrosis stage F4, i.e. cirrhosis over 14 kRa. For the optimal cut-off for advanced fibrosis, we accept 9.9 kPa (stage F3 and F4), and for liver density below 7.9 kPa absence of significant fibrosis (F0-1).

In the group studied by us, patients with no evidence of fibrosis - F0 and a fibroscan value below 5.5 kPa – were 39 patients (42.85%), of which 17 men (43.59%) and 22 women (56.41 %) (Fig. 28). There were 21 patients (23.08%) with mild fibrosis F1 (5.5-7 kRa), of which 14 were men (66.67%) and 7 were women (33.33%). With F2 fibrosis (up to 9.9 kRa) were 15 patients (16.48%), of which 7 men (46.67%) and 8 women (53.33%). With advanced fibrosis F3 (over 10 kRa to 14 kRa) there were 6 patients (16.48%), of which 2 men (33.33%) and 4 women (66.67%). There were 10 patients (11%) with evidence of F4 fibrosis corresponding to cirrhosis (over 14 kPa), of which 5 men (50%) and 5 women (50%) (Fig. 29).

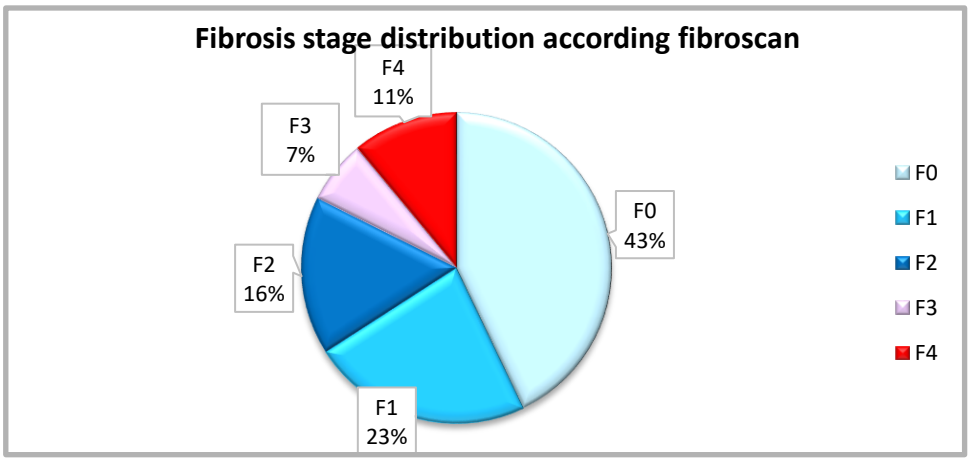
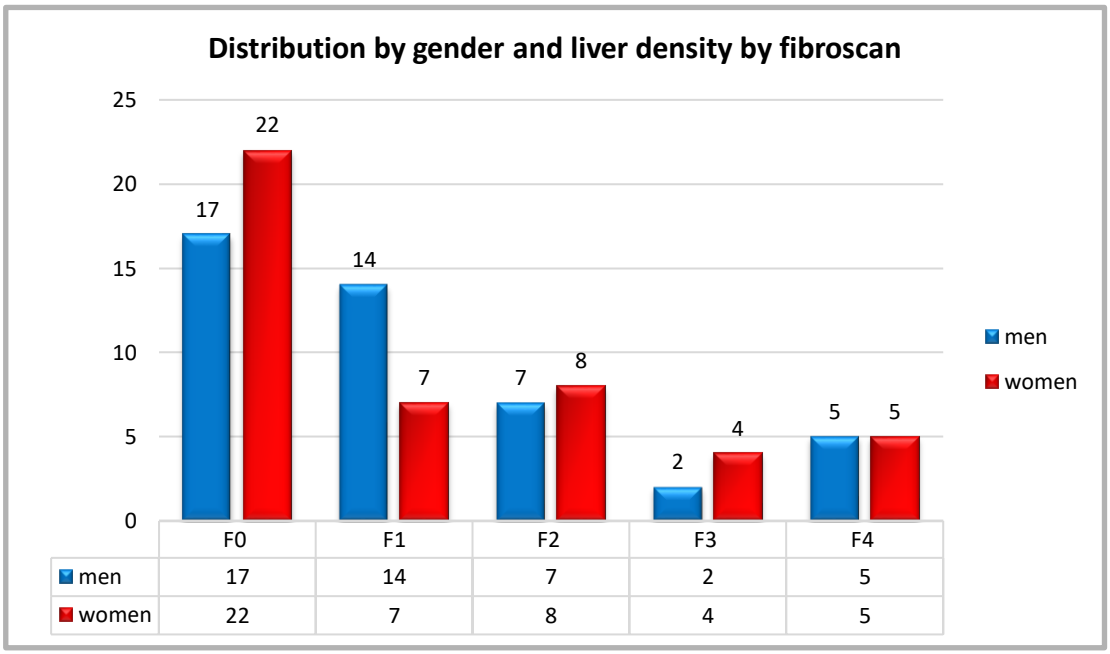


Fig. 28 Fibrosis stage distribution according to liver density measured with fibroscan



	F0	F1	F2	F3	F4
men	17	14	7	2	5
women	22	7	8	4	5

Fig. 29 Distribution by gender and liver density by fibroscan results

The distribution of fibrosis by gender was as follows: in men (n=45), F0 – 17 (37.78%), F1 – 14 (31.11%), F2 – 7 (15.56%), F3 – 2 (4.44%), F4 – 5 (11.11%), in females (n=46), F0 – 22 (47.82%), F1 – 7 (15.22%), F2 – 8 (17, 39%), F3 – 4 (8.7%), F4 – 5 (10.87%).

In our study group of patients, those with absent or mild fibrosis predominated (F0 and F1, 66%), which is consistent with literature data on the characteristics and clinical course of

the disease worldwide. However, patients with advanced fibrosis and cirrhosis, which are the main predictors of an aggressive course of the disease, also represent a significant proportion of the studied group (F3 and F4, 17.6%), which requires their active search and more active monitoring in view of the high frequency of liver-related morbidity and CVD morbidity and the need of treatment. The proportion of patients with significant F2 fibrosis is 16.4%. These patients are in a reversible phase of liver changes and would benefit from dietary and exercise measures and treatment of metabolic disorders. The performed study of liver density is of great importance for the clinical assessment of patients and their risk stratification and determination of further behavior.

4.2.14. Results of the study scores for fibrosis

Fibrosis stage 4 (FIB-4) - was calculated based on the values of age, AST, ALT and platelets according to the relevant formula. FIB-4 < 1.30 rejects advanced fibrosis (F0-F1), FIB-4 between 1.30 and 2.67 – gray area, and FIB-4 > 2.67 predicts advanced fibrosis (F3 – F4). The score was determined in 146 patients (men – 63 and women – 83), with the average value being 1.20±0.73, minimum 0.31 and maximum 5.37.

Values lower than 1.3 correspond to the absence of advanced fibrosis, i.e. F0-F1 were found in 99 patients (67.8%), males 45 and females 54. Values above 2.67, which are predictive of the presence of advanced fibrosis, i.e. F3-F4 are 7 (4.8%), males 2 and females 5.

Indeterminate score in the gray area, ie. between 1.3 and 2.67, corresponding to moderate fibrosis had 40 patients (27.4%), men 16 and women 24 (table 23 and fig. 30).

FIB-4 / fibrosis	men	women	Total	%
FIB4 <1,3, F0-F1,	45	54	99	67,8%
FIB4 1,3-2,67, F1-F2,	16	24	40	27,4%
FIB4 >2,67, F3-F4	2	5	7	4,8%
Total	63	83	146	100%

Table 23 Results of FIB-4 score - distribution by gender and stage of fibrosis

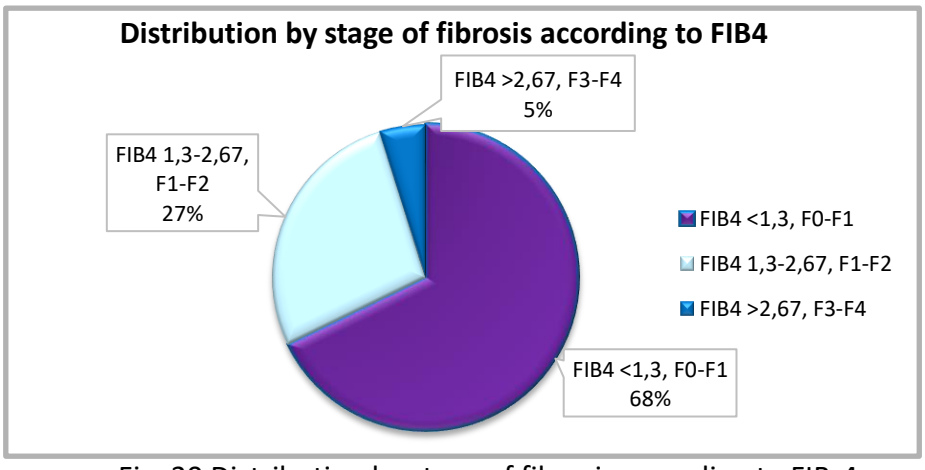


Fig. 30 Distribution by stage of fibrosis according to FIB-4

The negative predictive value for ruling out advanced fibrosis is higher than the positive predictive value, therefore non-invasive scores can be used as first-line risk stratification to rule out advanced disease.

NFS (NAFLD fibrosis score) was calculated based on the values of age, BMI, increased fasting glycemia or type 2 DM, AST, ALT, platelets and albumin. NFS <-1.455 - rejects significant fibrosis (F0-F2), NFS between ≤-1.455 and ≤ 0.675 - gray zone, NFS >0.675 - predictor of presence of significant fibrosis (F3-F4). The score was determined in 128 patients (men – 53 and women – 75), with the average value being -1.19±1.41, minimum -4.49 and maximum 2.58.

Values lower than -1.45 correspond to the absence of advanced fibrosis, i.e. F0-F1 were found in 52 patients (40.62%), males 22 and females 30. Indeterminate score in the gray zone, i.e. between -1.45 and 0.675, corresponding to moderate fibrosis had 62 patients (48.44%), men 26 and women 36. Values above 0.675, which are predictive of the presence of advanced fibrosis, i.e. F3-F4 were found in 14 patients (10.94%), 5 males and 9 females (Table 24 and Fig. 31).

NFS / fibrosis	men	women	total	%
NFS <-1,45, F0-F1	22	30	52	40,62%
NFS -1,45-0,675, F1-F2	26	36	62	48,44%
NFS >0,675, F3-F4	5	9	14	10,94%
Total	53	75	128	100%

Table 24 NFS score results - distribution by gender and fibrosis stage

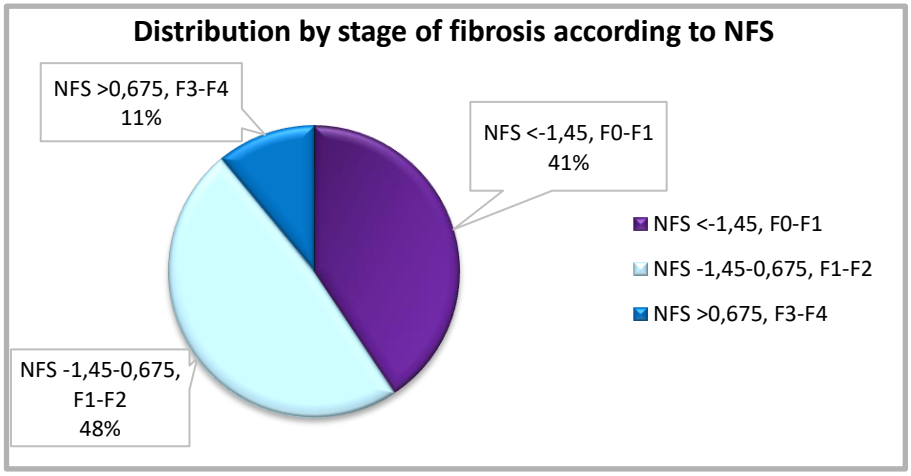


Fig. 31 Distribution by stage of fibrosis according to NFS

- *APRI score (AST to Platelet Ratio Index - APRI)* was calculated based on upper reference value of AST and platelet count. Values < 0.5 reject the presence of fibrosis; > 0.7 - suggest advanced fibrosis (F3 - F4), and > 1 (APRI 1) score - cirrhosis (F4). The latter is safer with an APRI score > 2, but with lower specificity. The score was determined in 146 patients (men – 63 and women – 83), with the average value being 0.34±0.32, minimum 0.07 and maximum 2.29.

Values lower than 0.5 correspond to the absence of advanced fibrosis, i.e. F0-F1 were found in 129 patients (88.3%), 59 men and 70 women (Table 25 and Fig. 32). Indeterminate score in the gray area, i.e. between 0.5 and 0.7, corresponding to moderate fibrosis had 3 patients (2.1%), male 1 and female 2. Values above 0.7, which are predictive of the presence of advanced fibrosis, i.e. F3-F4 were found in 13 patients (8.9%), men 3 and women 10. An APRI value above 2, which is a predictor of cirrhosis, was found in 1 woman with APRI=2.29, in whom by fibroelastography indeed, evidence of cirrhosis was present (F=24.5 kPa).

APRI / fibrosis	men	women	Total	%
APRI <0,5, F0-F1	59	70	129	88,3%
APRI 0,5-0,7, F1-F2	1	2	3	2,1%
APRI >0,7, F3-F4	3	10	13	8,9%
APRI > 2	0	1	1	0,7%
Total	63	83	146	100%

Table 25 Distribution by fibrosis stage and gender by APRI

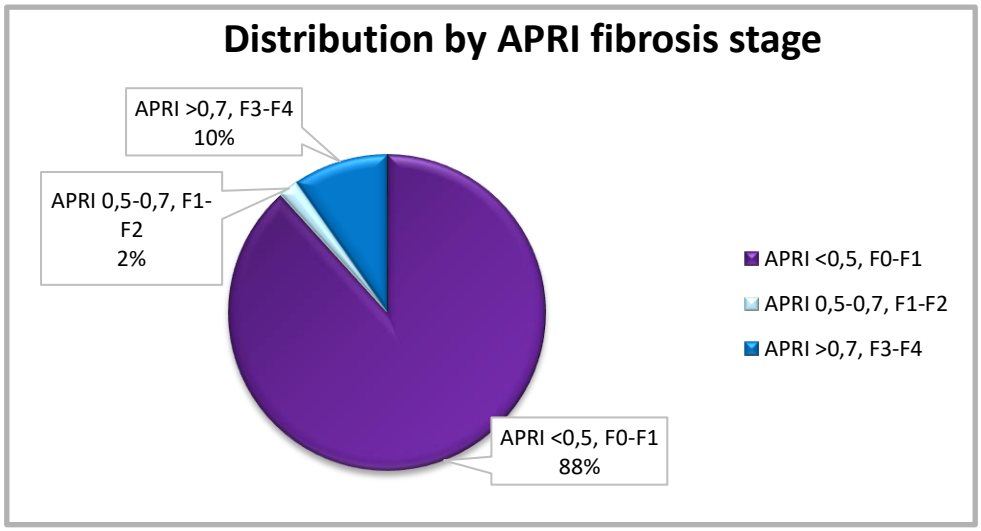


Fig. 32 Distribution by APRI fibrosis stage

- *BARD score* was calculated based on BMI, AST/ALT and the presence of DM. It is an easy-to-calculate score in which the possible number of points ranges from 0 to 4. According to the results of Harrison et al, a BARD score of 0 and 1 has an NPV of 96% for advanced fibrosis. The score was calculated in 130 patients (55 men and 75 women), with a value of 0 or 1 found in 19 patients (11 men and 8 women). Value 2 in 32 patients (13 men and 19 women). Value 3 in 50 patients (21 men and 29 women). Value 4 in 29 patients (10 men and 19 women). According to the results thus presented, compared to this score, it is established that only 14.61% do not have advanced fibrosis, i.e. have a score of 0 or 1 point.

- *AST/ALT ratio* - values above 1 are predictive of advanced fibrosis. The AST/ALT ratio is also used in other score systems. In the group studied by us (n=148), 52 patients (18 men and 34 women) had a value above 1, i.e. According to this score, 35% of the studied group had advanced fibrosis.

- *FAST score* - FibroScan-AST score (FAST score), includes liver density assessed by TE, CAP and AST value. A value ≤0.35 excludes the presence of significant inflammation and fibrosis. A value of 0.35-0.67 indicates active disease, with significant fibrosis (NASH + NAS≥4 + F≥2), and a value above ≥ 0.67 is associated with advanced disease.

FAST score was examined in 81 patients, of whom 41 were men and 40 were women. The average value found was 0.26±0.23, the minimum value was 0, the maximum value was 0.91. FAST score ≤0.35, speaking of the absence of inflammation and fibrosis, was found in 57 patients (70.37%), of which 29 were men and 28 were women. FAST score 0.35-0.67, which is a predictor of active disease and significant fibrosis, was found in 18 patients (22.22%), of which 10 were men and 8 were women. FAST score ≥ 0.67, establishing advanced disease, was found in 6 patients (7.41%), of which 2 were men and 4 were women (Table 26 and Fig. 33). FAST score is a mathematical model for non-invasive identification of NASH patients with significant fibrosis and activity. Therefore, this score provides a noninvasive way to identify patients at risk of progressive disease.

NFS / Fibrosis, activity	men	women	total	%
FAST score ≤0,35, no fibrosis and activity	29	28	57	70,37%
FAST score 0,35-0,67, active disease, significant fibrosis	10	8	18	22,22%
FAST score ≥ 0,67 advanced disease	2	4	6	7,41%
Total	41	40	81	100%

Table 26 Distribution by gender and disease severity (activity + fibrosis) according to the FAST score

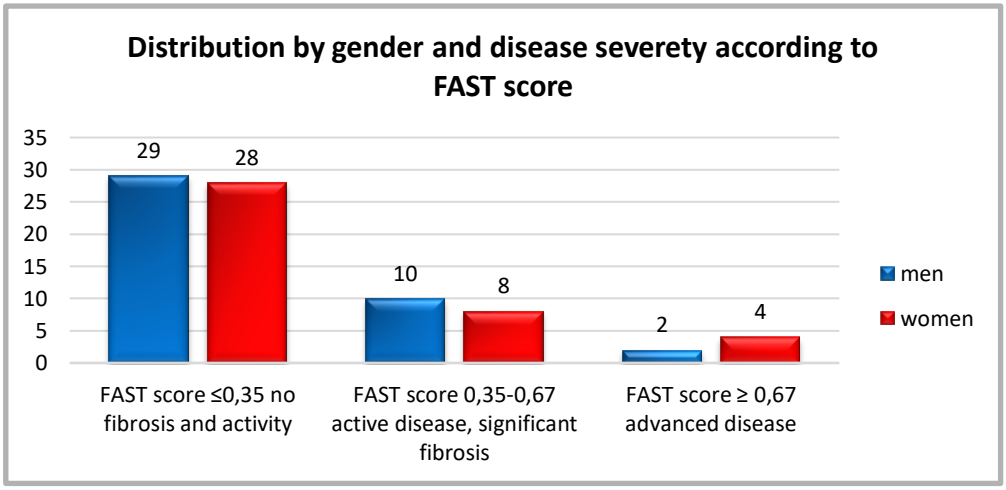


Fig. 33 Distribution by gender and disease severity (activity + fibrosis) according to the FAST score

4.2.15. Statistical analysis of the results of liver density measurement by fibroscan and its relation with the clinical characteristics of the patients, the defined indexes of fibrosis and comparison with the results of steatosis by CAP.

Correlation analysis was used to examine the relation between clinical indicators and establish the strength of their influence on the stage of fibrosis measured by fibroscan. Results of Pearson correlation analysis showed significant, moderately strong relation between liver density (F) and BMI (r=0.379, p=0.001). A strong correlation was also observed between F value and waist circumference (r=0.463, p=0.001). These correlation confirm that with an increase in the Body Mass Index and even more so in the waist circumference, the values of the liver density, also increase (table 27, fig. 34). Fibrosis is the main predictor of the progressive course of the disease.

Correlation Matrix									
			F- elastography (кPa)		**BMI=**		**Waist (cm)**	
F- elastography (кPa)	Pearson's r		—					
	p-value		—					
BMI=	Pearson's r		0.379	***	—			
	p-value		< .001		—			
Waist (cm)	Pearson's r		0.463	***	0.835	***	—	
	p-value		< .001		< .001		—	
Note. * p < .05, ** p < .01, *** p < .001								

Table 27 Correlation between BMI, abdominal circumference and fibroscan liver density

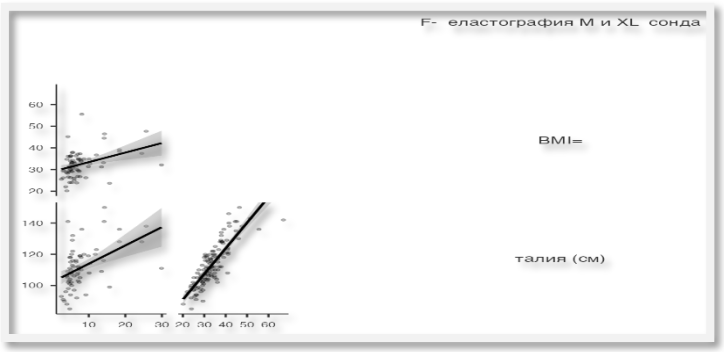


Fig. 34 Graphic presentation of the correlation dependence between BMI, abdominal circumference and liver density by fibroscan

Correlation analysis according to the Spearman method was used to study the dependencies between the categorical clinical indicators - stage of HD, presence of DM, presence of metabolic syndrome versus stage of fibrosis from fibroscan (table 28 and fig. 35). A moderately strong correlation was found between the stage of fibrosis and the presence of type 2 DM (rho=0.388, p=0.001). Such was not found with the presence of metabolic syndrome (rho=0.140, p=0.187), which is probably explained by the nature and size of the sample. Regarding the correlation between the presence of HD and the stage of fibrosis, the determined dependence is weak (rho=0.267, p=0.010).

Correlation Matrix										
				F (кPa)		DM		MS		
F (кPa)		Spierman's rho		—						
		p-value		—						
DM		Spierman's rho		0.388	***	—				
		p-value		< .001		—				
MS		Spierman's rho		0.140		0.308	***	—		
		p-value		0.187		< .001		—		
Note. * p < .05, ** p < .01, *** p < .001										

Table 28 Correlation between presence of DM, MS and liver density

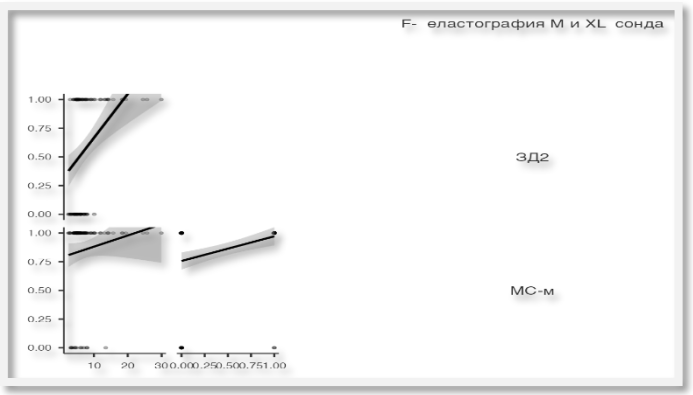


Fig. 35 Graphical presentation of the correlation between the presence of DM, MS and liver density

A Pearson correlation analysis was also carried out between the values of liver density and laboratory parameters - AST, ALT, GGT, platelets and triglycerides (table 29). The analysis showed a statistically significant but weak correlation only between AST values and fibrosis (rho=0.250, p=0.017).

Correlations							
		AST	ALT	GGT	Tr	Tg	F
AST	Pearson Correlation	1	.853**	.489**	-.085	-.032	.250*
	P		.000	.000	.308	.712	.017
	N	147	147	109	146	139	90
ALT	Pearson Correlation	.853**	1	.487**	-.099	-.020	.176
	P	.000		.000	.233	.813	.097
	N	147	147	109	146	139	90
GGT	Pearson Correlation	.489**	.487**	1	-.121	.026	.152
	P	.000	.000		.211	.795	.284
	N	109	109	109	109	106	52
Tr	Pearson Correlation	-.085	-.099	-.121	1	.153	-.139
	P	.308	.233	.211		.071	.195
	N	146	146	109	146	139	89

Tg	Pearson Correlation	-.032	-.020	.026	.153	1	-.078
	P	.712	.813	.795	.071		.488
	N	139	139	106	139	139	82
F	Pearson Correlation	.250*	.176	.152	-.139	-.078	1
	P	.017	.097	.284	.195	.488	
	N	90	90	52	89	82	91

Table 29 Correlation between the values of F - liver density from fibroscan and laboratory parameters - AST, ALT, GGT, platelets, triglycerides.

In order to compare the fibrosis scores FIB-4 score, NAFLD fibrosis score and APRI to the liver density results from elastography in fibroscan we used Pearson correlation analysis (Table 30, Fig. 36). The results of the analysis showed that the F values of fibrosken increased in parallel with the increase in scores. For FIB-4, the established correlation is moderately strong (r=0.365, p=0.001). The APRI data are similar, where the correlation with liver density values on fibroscan is also moderately strong (r=0.327, p=0.002). The highest correlation dependence was observed between the NAFLD fibrosis score and the fibroscan result (r=0.432, p=0.001).

Correlation Matrix											
			F κPa		**FIB-4 index**		**NAFLD fibrosis score**		**APRI**	
F κPa		Pearson's r	—							
		p-value	—							
FIB-4 index		Pearson's r	0.365	***	—					
		p-value	< .001		—					
NAFLD fibrosis score		Pearson's r	0.432	***	0.545	***	—			
		p-value	< .001		< .001		—			
APRI		Pearson's r	0.327	**	0.702	***	0.178	*	—	
		p-value	0.002		< .001		0.045		—	
Note. * p < .05, ** p < .01, *** p < .001										

Tabl. 30 Correlation between fibroscan fibrosis and fibrosis scores - FIB-4, NAFLD fibrosis score and APRI

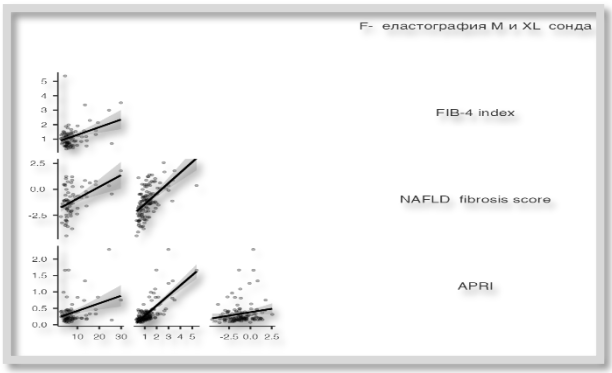


Fig.36 Graphical presentation of the correlation between fibroscan fibrosis and fibrosis scores

One-way ANOVA analysis was performed to determine whether there were statistically significant differences between the four fibrosis groups as determined by mean fibroscan and FIB-4 liver density values, NAFLD fibrosis score, and APRI (Tables 31 and 32). Statistically significant differences were found between the four fibrosis stages in the determined fibrosis indexes, for NAFLD fibrosis score (F=3.562, p=0.011), and for FIB-4 (F=2.812, p=0.030). For APRI only, the comparison between fibrosis groups was not statistically significant (F=2.193, p=0.77).

ANOVA

		Sum of Squares	df	Mean Square	F	Ниво на значимост
NADFLD	Between Groups	24.077	4	6.019	3.562	.011
	Within Groups	111.543	66	1.690		
	Total	135.620	70			
FIB4	Between Groups	5.663	4	1.416	2.812	.030
	Within Groups	42.288	84	.503		
	Total	47.951	88			
APRI	Between Groups	1.069	4	.267	2.193	.077
	Within Groups	10.233	84	.122		
	Total	11.301	88			

Table 31 Comparison by stages of fibrosis from fibroscan and NAFLD fibrosis score, FIB-4 and APRI

Count		Mean value	Std. deviation	Std. error	95% Confidence interval	Minimal values		Min.	Max.
						lower	upper		
NAFLD fibrosis score	под 5,5 кРа	30	-1.5770	1.43665	.26229	-2.1135	-1.0405	-4.49	1.24
	F1	14	-1.4543	1.04294	.27874	-2.0565	-.8521	-3.61	.74
	F2	13	-.9385	1.45802	.40438	-1.8195	-.0574	-3.81	1.21
	F3	5	-.4400	1.03012	.46068	-1.7191	.8391	-1.82	.85
	F4	9	.1067	.98698	.32899	-.6520	.8653	-1.61	1.79
	Total	71	-1.1424	1.39191	.16519	-1.4719	-.8129	-4.49	1.79
FIB4	под 5,5 кРа	39	1.0769	.78639	.12592	.8220	1.3318	.31	5.37
	F1	19	1.0068	.43998	.10094	.7948	1.2189	.37	1.98
	F2	15	1.0260	.39978	.10322	.8046	1.2474	.40	1.67
	F3	6	1.6767	.90608	.36991	.7258	2.6275	.83	3.36
	F4	10	1.7150	.99786	.31555	1.0012	2.4288	.53	3.51
	Total	89	1.1655	.73817	.07825	1.0100	1.3210	.31	5.37
APRI	под 5,5 кРа	39	.3138	.28612	.04582	.2211	.4066	.07	1.66
	F1	19	.3500	.34039	.07809	.1859	.5141	.07	1.67
	F2	15	.2740	.14387	.03715	.1943	.3537	.09	.62
	F3	6	.6167	.44329	.18097	.1515	1.0819	.20	1.34
	F4	10	.5790	.64669	.20450	.1164	1.0416	.14	2.29
	Total	89	.3651	.35836	.03799	.2896	.4405	.07	2.29

Table 32 Comparison by fibroscan fibrosis groups and fibrosis index values - NAFLD fibrosis score, FIB-4 and APRI

We used Tukey's Post Hoc Test to determine exactly which fibrosis groups had a significant difference in fibrosis index values. The mean values of the NAFLD fibrosis score, FIB-4 and APRI in the four Fibroscan fibrosis stages were compared. Differences between absent and mild fibrosis stages were found to be statistically significant, relative to F3 and F4.

In order to investigate the existence of a relation between the increase in steatosis and the increase in fibrosis stages, we performed an ANOVA comparative analysis by groups (Table 33, Fig. 37). The comparison between CAP and fibrosis stage found statistically significant differences, i.e. correlation of higher CAP value versus higher F value (F=5.270, p=0.001). The detailed differences between the groups can be seen in the Multiple Comparisons table (Table 34). For F4 versus the group without fibrosis p=0.027, for F4 versus F1 p=0.008, and versus F2 p=0.083. Only for F4 compared to F3 p=0.965, i.e. the difference was not significant between

the CAP values in the advanced fibrosis groups, which is explained by the similar high CAP value in both groups. For F1 vs. F2 p=0.921, and for F2 vs. F3 p=0.58.

ANOVA

CAP db/m

	Sum of Squares	df	Mean Square	F	p-level
Between Groups	39446,108	4	9861,527	5,270	,001
Within Groups	147817,928	79	1871,113		
Total	187264,036	83			

Table 33 Comparison between CAP and stages of fibrosis

Multiple Comparisons

Dependent Variable: CAP db/m

Tukey HSD

(I) stage of fibrosis	(J) stage of fibrosis	Mean Difference (I-J)	Std. Error	p-level	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
absent	F1	12,642	12,231	,839	-21,50	46,79
	F2	-,409	13,768	1,000	-38,85	38,03
	F3	-68,024*	22,659	,029	-131,28	-4,77
	F4	-50,649*	16,719	,027	-97,33	-3,97
F1	No	-12,642	12,231	,839	-46,79	21,50
	F2	-13,051	15,744	,921	-57,01	30,90
	F3	-80,667*	23,911	,010	-147,42	-13,91
	F4	-63,292*	18,380	,008	-114,61	-11,98
F2	No	,409	13,768	1,000	-38,03	38,85
	F1	13,051	15,744	,921	-30,90	57,01
	F3	-67,615	24,733	,058	-136,66	1,43
	F4	-50,240	19,438	,083	-104,51	4,03
F3	No	68,024*	22,659	,029	4,77	131,28
	F1	80,667*	23,911	,010	13,91	147,42
	F2	67,615	24,733	,058	-1,43	136,66
	F4	17,375	26,489	,965	-56,58	91,33
F4	No	50,649*	16,719	,027	3,97	97,33
	F1	63,292*	18,380	,008	11,98	114,61
	F2	50,240	19,438	,083	-4,03	104,51
	F3	-17,375	26,489	,965	-91,33	56,58

Table 34 Comparison between CAP and stage of fibrosis

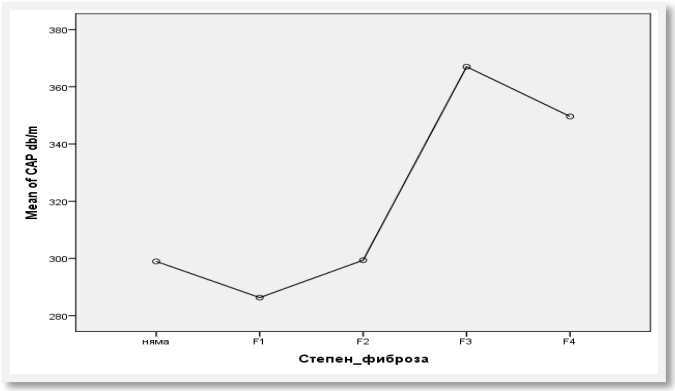


Fig. 37 Graphic presentation of the relationship between CAP and the stage of fibrosis

We performed a comparative analysis between the patients without fibrosis and the four groups with fibrosis assessed by fibroscan in terms of BMI, abdominal circumference in centimeters, ALT and AST (tables 35 and 36). Descriptive subgroup analysis found that the mean BMI in patients with absent fibrosis was lower compared with the mean in advanced fibrosis F3 and F4. Accordingly, for patients without fibrosis the mean value was 33.77, for F1 the mean BMI was 35.35, for F2 it was 33.68, for F3 it was 34.20, and for F4 it was 36.26. Also, the maximum BMI values were found in the group with the highest stage of fibrosis – F4. ANOVA analysis performed to determine whether there were statistically significant differences between fibrosis groups found that there was no statistically significant difference between the four fibrosis stages in terms of BMI (F=0.279, p=0.890). This is explained by the fact that the majority of patients studied by us were in the group with absent or no significant fibrosis (66%).

Descriptive analysis regarding waist circumference showed its gradual increase with increasing fibrosis stage. Accordingly, for patients without fibrosis the mean value was 105.7 cm, for F1 the mean value of waist circumference was 111.25 cm, for F2 it was 113 cm, for F3 it was 115 cm, and for F4 it was 127.5 cm, and again the maximum values are observed at F4. ANOVA analysis performed to determine whether there were statistically significant differences between fibrosis groups found that there was a statistically significant difference between the four stages of fibrosis in terms of waist circumference (F=5.917, p=0.000). For F4 versus the group without fibrosis p=0.000, for F4 versus F1 p=0.025, and versus F2 p=0.049. Only for F4 compared to F3 p=0.329, i.e. the difference is not significant.

Subgroup ANOVA analysis found no statistical significance between ALT value and fibrosis stage (F=0.949, p=0.440). Regarding AST, descriptive analysis between groups showed lower mean values that were within reference limits for F0, F1, F2 of 33, 35 and 27 U/l, respectively. For F3 and F4, respectively, the average values of AST were 53.16 U/l and 51.6 U/l, i.e. above the upper reference value for both groups of advanced fibrosis. However, the comparative analysis did not show statistical significance (F=1.471, p=0.218), which is probably explained by the significantly smaller number of patients with advanced fibrosis F3 and F4.

Multiple Comparisons

Tukey HSD

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
BMI=	no	25	33,77	5,341	1,068	31,57	35,98	25	45
	F1	13	35,35	7,303	2,025	30,94	39,76	27	51
	F2	10	33,68	5,558	1,757	29,70	37,65	25	41
	F3	5	34,20	8,257	3,693	23,95	44,46	26	46
	F4	10	36,26	11,977	3,788	27,69	44,83	25	67
	Total	63	34,51	7,215	,909	32,70	36,33	25	67
Waist (cm)	no	30	105,700	12,1120	2,2113	101,177	110,223	85,0	141,0
	F1	12	111,250	10,6012	3,0603	104,514	117,986	92,0	132,0
	F2	13	112,846	10,7071	2,9696	106,376	119,316	94,0	136,0
	F3	5	114,800	6,6106	2,9563	106,592	123,008	108,0	123,0
	F4	9	127,444	16,1564	5,3855	115,026	139,863	99,0	150,0
	Total	69	111,507	13,5610	1,6326	108,250	114,765	85,0	150,0
ALT	no	40	38,638	31,9736	5,0555	28,412	48,863	7,5	176,0
	F1	19	62,268	97,0545	22,2658	15,490	109,047	10,0	448,0
	F2	15	41,933	34,2104	8,8331	22,988	60,878	12,0	146,0
	F3	6	69,000	46,0869	18,8149	20,635	117,365	21,0	120,0
	F4	10	64,500	80,8321	25,5614	6,676	122,324	19,0	283,0
	Total	90	49,073	58,8679	6,2052	36,744	61,403	7,5	448,0
ALT	No	40	33,055	27,2369	4,3065	24,344	41,766	6,5	125,0
	F1	19	35,916	34,7112	7,9633	19,186	52,646	15,0	172,0
	F2	15	27,467	12,5007	3,2277	20,544	34,389	13,0	55,0
	F3	6	53,167	33,0661	13,4992	18,466	87,867	19,0	87,0
	F4	10	51,600	50,7460	16,0473	15,299	87,901	19,0	178,0
	Total	90	36,129	31,2817	3,2974	29,577	42,681	6,5	178,0

Table 35 Descriptive analysis by groups of fibrosis stages and BMI, waist circumference, AST, ALT

ANOVA

		Sum of Squares	df	Mean Square	F	p-level
BMI=	Between Groups	60,905	4	15,226	,279	,890
	Within Groups	3166,476	58	54,594		
	Total	3227,381	62			
waist (cm)	Between Groups	3375,982	4	843,995	5,917	,000
	Within Groups	9129,265	64	142,645		
	Total	12505,246	68			
ALT	Between Groups	13191,308	4	3297,827	,949	,440
	Within Groups	295231,888	85	3473,316		
	Total	308423,196	89			
AST	Between Groups	5639,594	4	1409,898	1,471	,218
	Within Groups	81450,611	85	958,242		
	Total	87090,205	89			

Table 36 Comparative analysis by groups of fibrosis stages and BMI, abdominal circumference, AST, ALT

4.2.16. Cytokeratin 18 test results

Cytokeratin 18 (CK18) fragments are markers of hepatocyte apoptosis and are formed upon cell death, as a result of the action of the enzyme caspase 3. As the only marker of apoptosis/necrosis and an indicator of liver inflammation, it is assumed that CK18 can be applied to distinguish simple non-progressive steatosis from steatotic hepatitis in NAFLD cases. We quantitatively measured the levels of total CK18 in the serum by "sandwich" ELISA method, which uses specific antibodies - anti-Human Cytokeratin 18 (Millipore, product number RAB1408). The measurement units of the obtained results are in ng/ml. According to the kit manufacturer's instructions, a reference value of 5 ng/ml was taken. This value is derived based on a study of healthy subjects during testing of normal serum samples in the validation of the test. A team from the University School of Medicine, Van, TURKEY, using cytokeratin 18 with the same units of measurement, suggested a reference value of 3.1 ng/ml. In this study we will stick to the value suggested by the manufacturer. Cytokeratin 18 (CK18) in serum was studied in 61 individuals 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 of 79 years. The measured mean value was 3.01±10.42 ng/ml, with a minimum value of 0.1 ng/ml and a maximum value of >80 ng/ml.

The characteristics of the studied group with cytokeratin 18 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 is 115.19±12.83, for men 119.34±13.01, and for women 112.68±12.21 (table 37). Of all 61 patients examined, 47 (77.04%) had evidence of registered HBV, and 14 did not have elevated BP values. In 20 patients (32.78%) DM type 2 was established, respectively 41 patients (67.22%) had no data on DM. In 8 (13.11%) of the patients, the full 3 acceptance criteria for the presence of metabolic syndrome are missing, and 1 or 2 risk factors are present.

	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

Table 37 Characteristics of patients tested for cytokeratin 18 in terms of age, BMI and abdominal circumference

Laboratory tests in the group showed an increase in ALT above normal. in 11 patients, with the maximum value being 146 U/l and the minimum being 5 U/l, mean value 32±32.09 U/l. The average value of AST was 28.78±19.94 U/l, with a maximum of 114 U/l and a minimum of 13.4 U/l. Increase of ASAT above u.l.n. we found in 6 patients. GGT is with u.l.n. different in both sexes, therefore the average value in men is 39.69±22.14 U/l, with a min. 12 U/l and max. 122 U/l, an increase in only 1 patient. In women, the mean GGT value was 44.39±43.98 U/l, min. 8 U/l and max. 178 U/l, as an increase was observed in 13 patients. Average value of triglycerides is 2.13±1.01 mmol/l, min. 1.07 mmol/l and max. 4.92 mmol/l, as an increase above u.l.n. was observed in 13 patients (Table 38). Albumin and platelet values were within reference limits in all patients.

	AST U/l	ALT U/l	GGT U/l мъже/жени	Tg mmol/l
Mean vealu	28,78	32	39,69/44,39	2,13
Standart deviation	19,94	32,09	22,14/43,98	1,01
Minimum value	13,4	5	12/8	1,07
Maximum value	114	114	122/178	4,92
Count (↑ value)	6	11	14	13

Table 38 Characteristics of patients tested for cytokeratin 18 in terms of AST, ALT, GGT, Tg

All patients (n=61) had sonographic evidence of steatosis, respectively mild in 11 patients, moderate in n=23 and severe in n=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. NAFLD fibrosis score has 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%), with a moderate fibrosis score between -1.45 and 0.675 there were 29 patients (47.5%), with advanced fibrosis there were 6 patients (9.8%).

Among the studied group, normal values of CK 18 were found in 55 patients (90.17%), and an increase in CK 18 above 5 ng/ml, indicating the presence of steatohepatitis, was found in 6 patients (9.83%), of which 2 males and 4 females (Fig. 38).

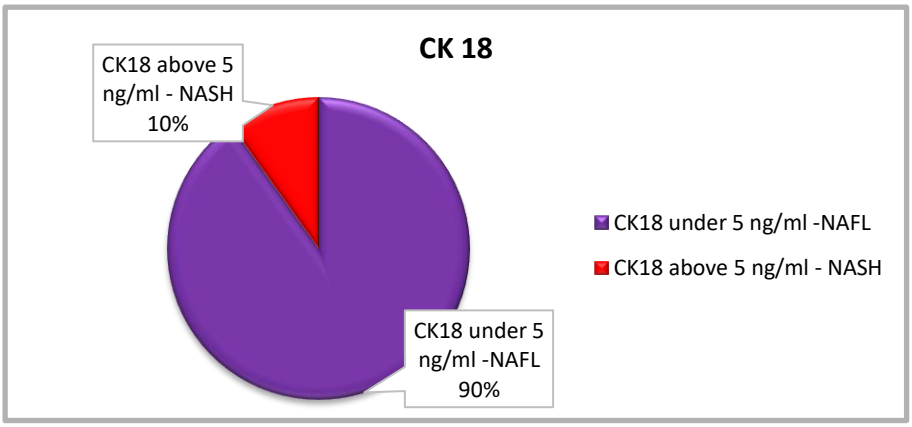


Fig. 38 Distribution by elevated value of SC18 – NASH/NAFL

At values of cytokeratin 18 above 5 ng/ml (u.l.n.), we accepted a diagnosis of steatohepatitis. The average age of patients with steatohepatitis was 56.5±11.41 years, minimum 41 years and maximum 73 years. Average value of BMI was 29.04±2.88, four were overweight BMI-25.14; 28.34; 29.24 and 29.40, and the rest have I degree of obesity - 32.61 and 32.89. All had evidence of visceral obesity, with increased waist circumferance corresponding to a significantly increased risk, in men average waist circumference - 114±.4.24 cm, and in women - 102.25±4.57 cm. Insulin resistance with increased HOMA-index above 2.5 was found in all patients. HD was proven in 5 of the patients (84%), and in 1 there was no evidence of hypertensive disease (the youngest of the examined group of patients). 3 have known diabetes and the remaining 3 have normal fasting blood sugar.

AST was elevated in 4 patients, ALT only in 3 patients, and GGT elevation was also observed in 4 of the patients. An increase in triglycerides above 1.7 mmol/l was also observed in 4 patients. All have normal albumin and platelet values. In 3 of these patients, the steatosis was estimated by the ultrasound examination as 2 grade, and in the remaining 3 it was estimated as 3 grade, and in all of them there was hepatomegaly with the average longitudinal size of the right liver lobe according to RMCL (right medioclavicular line) - 163.8±163 mm, maximum 176mm and minimum 150mm, no splenic enlargement. Estimated fibrosis scores - FIB-4 and NAFLD fibrosis score are in the gray zone, indicating moderate fibrosis. The mean value of FIB-4 was 1.77±0.48 and the NAFLD fibrosis score was -1.36±1.09. Patients with elevated cytokeratin 18 do not have advanced fibrosis.

4.2.17. Statistical analysis of the results of cytokeratin 18 as a marker for liver inflammation and its correlation with the clinical characteristics of patients, defined scores of fibrosis and comparison with the degree of steatosis and changes in laboratory parameters.

Correlation analysis according to the Pearson method was used to study the relationships between clinical indicators and establish the strength of their influence on cytokeratin values (Table 39, Fig. 39). The results of the correlation analysis showed the presence of a moderately strong positive relation between the level of triglycerides and CK 18 (r=0.318, p=0.012), indicating an increase in the value of triglycerides in parallel with the values of CK 18. Such a relation was not found regarding the increase in BMI and waist circumference on the one hand and the increase in CK 18 on the other.

Correlation Matrix

		CK 18		BMI=		waist (cm)		Tg	
CK 18	Pearson's r	—							
	p-value	—							
BMI=	Pearson's r	-0.087		—					
	p-value	0.507		—					
Waist (cm)	Pearson's r	-0.038		0.835	***	—			
	p-value	0.769		< .001		—			
Tg	Pearson's r	0.318	*	-0.029		0.022		—	
	p-value	0.012		0.748		0.810		—	

Note. * p < .05, ** p < .01, *** p < .001

Table 39 Correlation analysis between BMI, triglyceride level, increase in abdominal circumference and the level of CK18.

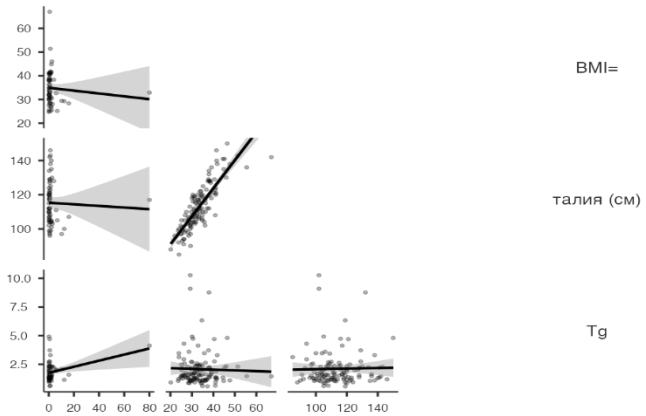


Fig. 39 Graphical representation of the results of the correlation analysis between BMI, waist, Tg and CK 18 level

The investigated correlation by the Spearman method between the presence of HD, DM and MS with the values of cytokeratin 18 did not give significant results (table 40). DM type 2 compared to CK18 (rho=0.082, p=0.532), HD compared to CK18 values (rho=0.176, p=0.174) and MS compared to CK18 (rho=-0.107, p=0.414).

Correlation Matrix											
		CK 18		DM		MS		HD			
CK 18	Spearman's rho	—									
	p-value	—									
DM t 2	Spearman's rho	0.082		—							
	p-value	0.532		—							
MS	Spearman's rho	-0.107		0.308	***	—					
	p-value	0.414		< .001		—					
HD	Spearman's rho	0.176		0.347	***	0.540	***	—			
	p-value	0.174		< .001		< .001		—			

Table 40 Correlation between the presence of HD, DM and MS with the values of SK18

Correlation analysis according to the Pearson method for the laboratory parameters AST, ALT, GGT, CRP and cytokeratin 18 did not show significant correlation (table 41).

Correlation Matrix											
		GGT									
		AST		ALT		GGT		CRP		CK 18	
AST	Pearson's r	—									
	p-value	—									
ALT	Pearson's r	0.853	***	—							
	p-value	< .001		—							
GGT	Pearson's r	0.489	***	0.487	***	—					
	p-value	< .001		< .001		—					
CRP	Pearson's r	0.170		0.084		-0.053		—			
	p-value	0.144		0.473		0.672		—			
CK 18	Pearson's r	0.159		0.095		0.117		-0.102		—	
	p-value	0.220		0.465		0.368		0.579		—	
Note. * p < .05, ** p < .01, *** p < .001											

Table 41 Pearson correlation between the values of AST, ALT, GGT,CRP and CK18

We considered this lack of correlation to be due to the lack of large variation in cytokeratin 18 values 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 CK 18 into

two groups - those with a normal CK 18 value and a group with elevated CK 8 above 5 ng/ml (table 41). The purpose of the analysis is to compare the average measured values of AST, ALT, GGT and CRP between the two groups (Table 42, Fig. 40).

The results showed that for AST the mean value was 25.9±16.4 U/l for the patients of the first group with normal cytokeratin 18 and for the second group with cytokeratin above 5 ng/ml the mean value was 54.93±31.06 U/l, therefore significantly increased AST values were observed in the second group, and these differences were statistically significant (t=-3.729, p=0.001). Regarding ALT, the mean value was 28.2±19.10 U/l for the patients of the first group with normal CK 18, and in the second group with cytokeratin above 5 ng/ml the mean value was significantly higher 66.63±55, 65 U/l, therefore significantly increased ALT values were observed in the second group, and these differences were statistically significant (t=-2.958, p=0.004) (Fig. 41).

The average value of GGT in the first group was 39.2±34.3, and in the group with elevated CK 18 it was 74.17±49.97, the difference being statistically significant (t=-2.266, p=0.027) (Fig. 42). Only with regard to CRP values, there are no statistically significant differences between the two groups (t=0.585, p=0.563).

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

Table 41 Independent Samples T-test for comparison of mean values of AST, ALT, GGT and CRP between normal and elevated groups CK18

Group Descriptives

	Group	N	Mean	Median	SD	SE
AST	under 5ng/ml	55	25.9	20.80	16.4	2.21
	above 5ng/ml	6	54.93	54.05	31.06	12.68
ALT	under 5ng/ml	55	28.2	19.10	26.9	3.63
	above 5ng/ml	6	66.63	55.65	54.20	22.13
GGT	under 5ng/ml	55	39.2	30.00	34.3	4.63
	above 5ng/ml	6	74.17	56.00	49.97	20.40
CRP	under 5ng/ml	28	12.3	4.88	26.6	5.02
	above 5ng/ml	4	4.45	3.72	2.95	1.47

Table 42 Descriptive description of the mean values of AST, ALT, GGT and CRP in the groups with normal and elevated CK 18

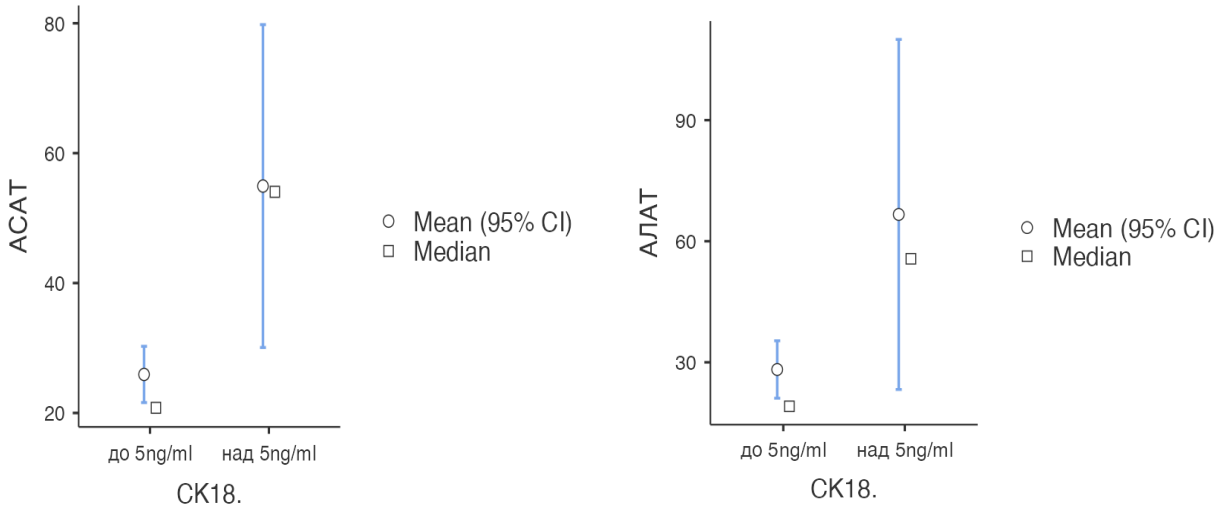


Fig. 41 Graphic representation of the comparison of the average values of AST and ALT, between the groups with normal and elevated CK 18

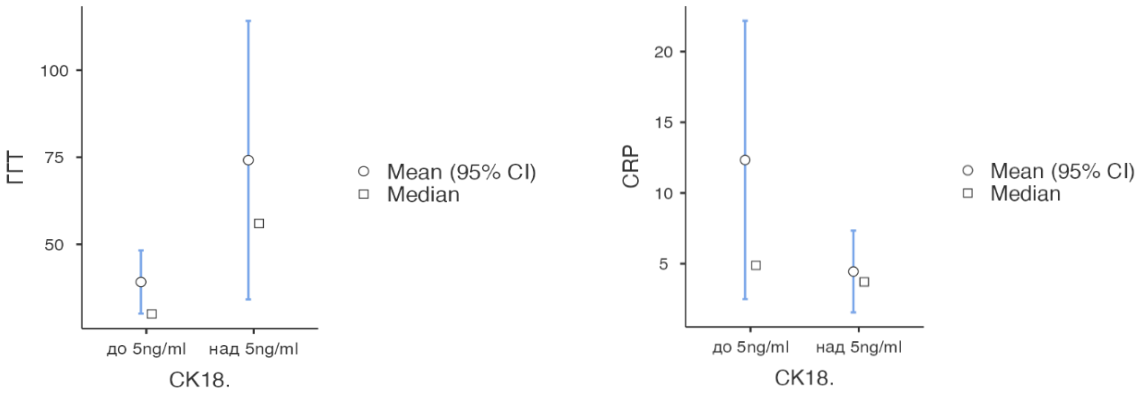


Fig. 42 Graphical representation of the comparison of the mean values of GGT and CRP between the normal and elevated groups CK18

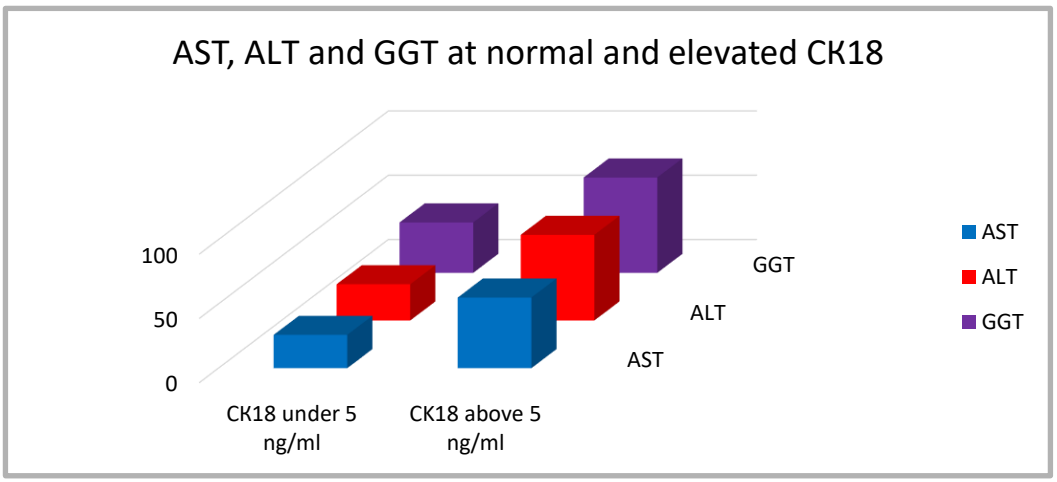


Fig. 40 Comparison of the average values of AST, ALT and GGT in groups with normal and elevated CK18

In order to investigate whether there is a correlation between the level of cytokeratin 18 and the degree of steatosis determined sonographically, we performed a correlation analysis, which did not show a statistical connection (rho=0.147, p=0.259) (table 43).

Correlation Matrix				
		цитокератин 18		**ехо-стеатоза степен**
цитокератин 18	Spearman's rho	—		
	p-value	—		
ехо-стеатоза степен	Spearman's rho	0.147		—
	p-value	0.259		—
Note. * p < .05, ** p < .01, *** p < .001				

Table 43 Correlation between degree of steatosis and values of CK18

We performed a correlation analysis between the SC18 level and the fibrosis scores - FIB-4 index, APRI and NAFLD fibrosis score, but we did not establish a statistically significant correlation (table 44).

Correlation Matrix						
	FIB-4 index		**APRI**		**NAFLD fibrosis score**	**CK 18**
FIB-4 index	Pearson's r	—				
	p-value	—				
APRI	Pearson's r	0.702	***	—		
	p-value	< .001		—		
NAFLD fibrosis score	Pearson's r	0.545	***	0.178	*	—
	p-value	< .001		0.045		—
CK 18	Pearson's r	0.034		0.145		-0.124
	p-value	0.792		0.265		0.343
Note. * p < .05, ** p < .01, *** p < .001						

Table 44 Correlation between fibrosis scores and values of CK18

With the help of Independent Samples T-test, we again divided the patients with tested CK 18 into two groups - those with a normal CK 18 value and a group with elevated CK 18 above 5 ng/ml and compared the mean values of FIB-4, APRI and NAFLD fibrosis score between the two groups (tables 45 and 46). The mean value of the NAFLD fibrosis score in the first group was -1.263±1.299, and in the group with elevated CK 18 it was -1.358±1.091, with

no statistically significant difference between the two groups (t=0.151, p=0.880). The mean value of the FIB-4 index in the first group was 1.204±0.692, and in the group with increased CK 18 it was 1.767±0.476, with no statistically significant difference between the two groups (t=-1.936, p=0.058). Accordingly, for APRI, the average value in the first group was 0.263±0.196, and in the group with elevated CK 18 it was 0.621±0.364, and only here was a statistically significant difference established (t=-3.872, p=0.001).

Independent Samples T-Test

		t	df	p
NAFLD fibrosis score	Student's t	0.151	59.0	0.880
FIB-4 index	Student's t	-1.936	59.0	0.058
APRI	Student's t	-3.872 [a]	59.0	< .001

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

Table 45 Comparison of the mean values of NAFLD fibrosis score, FIB-4 and APRI in the groups with normal and elevated CK18

Group Descriptives

	Group	N	Mean	Median	SD	SE
NAFLD fibrosis score	under 5ng/ml	55	-1.263	-1.299	1.483	0.2000
	above 5ng/ml	6	-1.358	-1.358	1.091	0.446
FIB-4 index	under 5ng/ml	55	1.204	0.973	0.692	0.0933
	above 5ng/ml	6	1.767	1.818	0.476	0.194
APRI	under 5ng/ml	55	0.263	0.197	0.196	0.0264
	above 5ng/ml	6	0.621	0.617	0.364	0.148

Table 46 Description of the mean values of NAFLD fibrosis score, FIB-4 and APRI in the groups with normal and elevated CK 18

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, are a very small proportion. The majority of patients either have no fibrosis or fall into the gray area with mild fibrosis. A larger sample of patients with different liver densities is likely needed to conclude whether elevated CK 18 levels correlate with higher-grade fibrosis. Regarding the APRI score, the patients are also without significant fibrosis, but this score is calculated on the basis of only two parameters, one of which is AST, and as we found out, in patients with

elevated CK 18, there is also a statistically significant increase in transaminases, which explains the described correlation with APRI.

4.3. Algorithm for screening and diagnosis of NAFLD

As a result of all the above established data, taking into account the importance of the components of the metabolic syndrome and the strength of their influence on the degree of steatosis and the stage of fibrosis and on the probable progressive course of NAFLD, having sufficient data on non-invasive biochemical and imaging methods for diagnosis and staging of the disease and considering the literature data, we propose the application of the following algorithm for screening and diagnosis of NAFLD (Fig.43).

We recommend that screening be performed among patients with risk factors and a high probability of NAFLD. These are patients with metabolic syndrome, patients with DM type 2, which suggests a more aggressive course of the disease, or patients with visceral obesity. Our study demonstrated that visceral obesity is a major predictor of underlying high-grade steatosis and fibrosis.

In the presence of the above risk factors, the first step is to calculate the steatosis scores - an easy, fast, affordable and reliable way of initial orientation for steatosis. For FLI data greater than 60 or HSI greater than 36, referral for abdominal ultrasound follows.

The first major imaging modality demonstrating hepatic steatosis is abdominal ultrasound, which provides a semiquantitative assessment of steatosis. When steatosis is proven, a hepatological screening is recommended to exclude a secondary cause of steatosis - a detailed history of ethanol intake, virological tests (anti HB cor total, HBsAg, anti HCV), copper exchange test (serum copper, ceruloplasmin), toxic reasons etc. In the absence of hepatic steatosis, but with known risk factors, follow-up of laboratory tests and re-screening after one year is recommended.

When a secondary cause of steatosis is ruled out, NAFLD is assumed and laboratory tests are performed. The minimum recommended tests are ALT, AST, GGT, albumin, platelets, lipid profile. To assess the presence of non-alcoholic steatohepatitis, cytokeratin 18 testing is recommended, if available.

The next recommended step is to assess the stage of fibrosis. Fibrosis indexes - FIB-4 and NAFLD fibrosis score - are calculated as the first line. Based on them, the first risk stratification of patients is possible.

A low risk of underlying progressive disease was assumed at FIB-4 < 1.3 and NFS < -1.455. In this case, follow-up laboratory tests should be recommended to patients and repeat screening after 1 year, while in the meantime a change in lifestyle is recommended - dietary regime and exercise regime, if necessary, treatment of metabolic disorders. At this stage, liver changes are reversible.

With a FIB-4 of 1.3 to 2.67 and an NFS of -1.455 to 0.675, patients are at moderate risk and further investigation to confirm fibrosis stage by elastography is recommended. With FIB-4 > 2.67 and NFS > 0.675, patients are stratified as high risk for progressive disease and confirmation of fibrosis stage by elastography is also recommended.

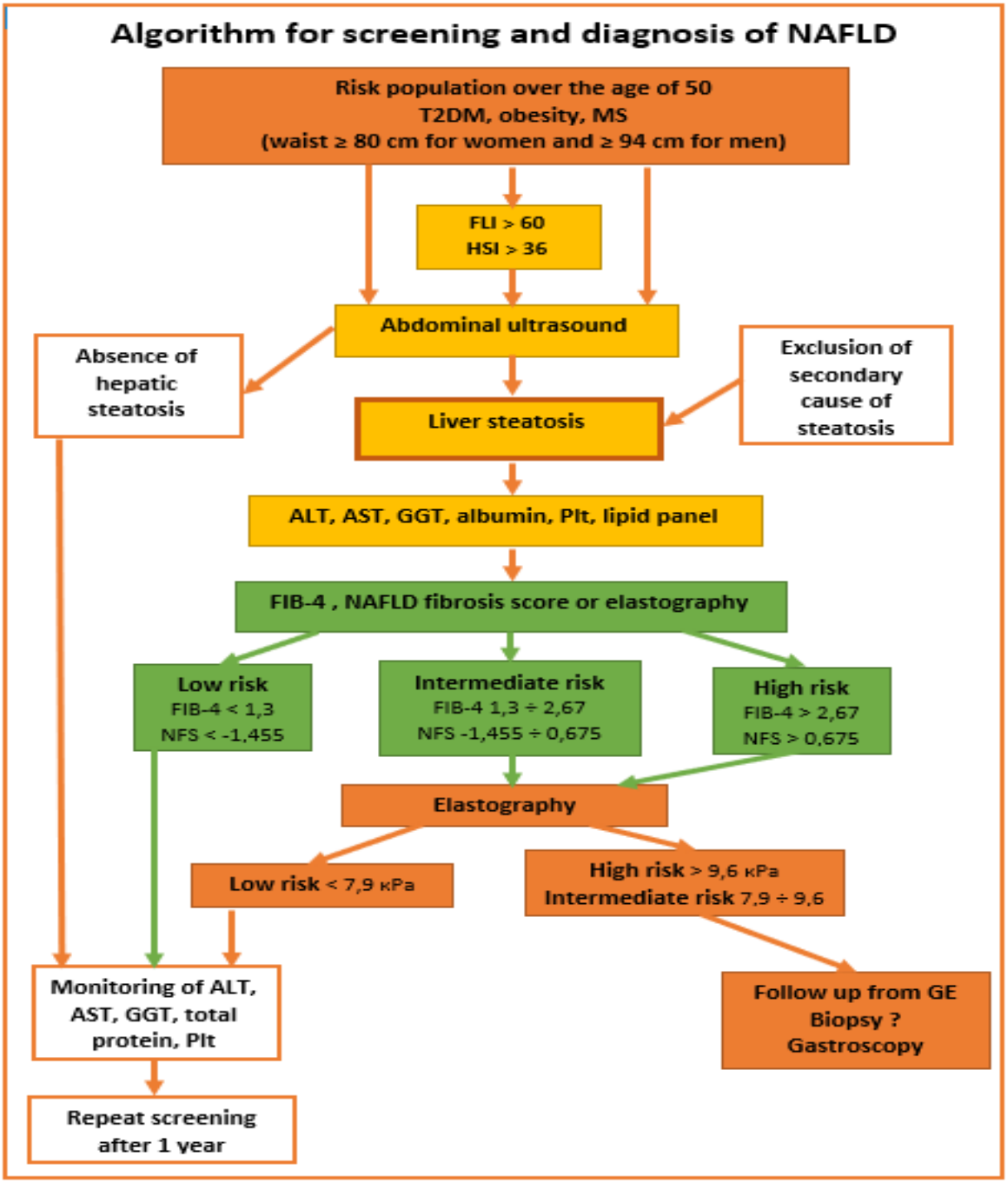


Fig. 43 Screening and diagnosis of NAFLD

A fibroscan elastography result below 7.9 kPa is considered to have no advanced fibrosis and is at low risk for progressive disease. They are subject to follow-up and re-screening after 1 year. A diet and exercise regimen are recommended, and if necessary, treatment of metabolic disorders.

A fibroscan elastography score between 7.9 kPa and 9.6 kPa stratifies patients at moderate risk of advanced disease. In case of elastography with a result above 9.6 kRa, they are correspondingly at high risk of advanced disease. In both cases, the patients have advanced fibrosis, which is why they need follow-up by a gastroenterologist, assessment of the need for a biopsy, assessment of performing upper endoscopy with a view to clarifying

the underlying cirrhosis and its staging. In addition to a recommendation for lifestyle changes and treatment of metabolic disorders, specific treatment is also necessary.

5. Discussion of the results

Non-alcoholic fatty liver disease (NAFLD) is a public health problem of global importance, affecting approximately one million individuals worldwide. Primary NAFLD is associated with insulin resistance and the presence of metabolic syndrome, which is a complex of risk factors. The incidence of NAFLD is increasing worldwide, in parallel with the increase in the incidence of components of the metabolic syndrome, in particular obesity and diabetes mellitus. NAFLD is the phenotypic hepatic manifestation of the metabolic syndrome. According to worldwide literature data, NAFLD is about to overtake other liver diseases in terms of morbidity, mortality and as an indication for liver transplantation. On the other hand, the presence of NAFLD is considered a predictor of the occurrence of cardiovascular and oncological diseases, as well as increased mortality from other extrahepatic causes. Considering the wide distribution, the possibility of a progressive course of the disease and the possibility of reversibility of the fatty infiltration process, we realize the importance of timely diagnosis of patients with NAFLD and identification of those with more advanced disease in terms of presence of inflammation and fibrosis.

In the current dissertation, we analyzed a group of patients from the Bulgarian population with data on non-alcoholic fatty liver disease in different stages of the disease and with different stages of fibrosis. We performed a complex assessment of the liver status by analyzing the results of the clinical examination, laboratory and imaging data.The clinical evaluation of the patients was made on the basis of a detailed history, including current complaints, assessment of accompanying diseases and risk factors, physical examination with assessment of anthropometric parameters, analysis of laboratory results and the results of imaging studies - ultrasound and fibroscan. Adequate clinical assessment of patients with NAFLD is of great importance given the different forms and stages of the disease, assessing different prognosis and treatment behavior. Moreover, subjective complaints among patients with NAFLD regardless of stage and prognosis are almost absent, which gives reason for patients to neglect the seriousness of the problem. This necessitates an intensive search and full clinical evaluation among at-risk groups to detect progressive disease.

In patients with NAFLD, in most cases, subjective complaints are absent or the complaints are non-specific. Historically, patients rarely report heaviness and discomfort in the right lower rib. In our studied group of 148 patients, only 9 reported such subjective complaints. Most often, steatosis was detected during a prophylactic ultrasound examination or during an ultrasound examination on the occasion of abdominal pain, upper or lower dyspeptic syndrome, associated with another pathology, which was the reason for the diagnosis in 32 of our patients. Another common reason for finding hepatic steatosis is the finding of elevated transaminases or cholestasis enzymes when conducting preventive laboratory tests or tests on another occasion. Thus, steatosis was diagnosed in 20 of our patients. In most of the patients in our group, steatosis was detected during preventive examination and targeted screening among patients with evidence of metabolic syndrome. In

a part of all these patients, advanced and active liver disease is established in opposition to the absence of subjective complaints and the lack of awareness of the presence of an existing liver problem. Therefore, active search for patients at risk of NAFLD among risk groups is essential to identify those with advanced disease.

Major predictors of NAFLD are components of the metabolic syndrome. Essential to the clinical evaluation of patients with NAFLD is the identification of risk factors. In the group studied by us, 62.84% of all patients have glycemic disorders, 47.29% of them have known type 2 DM, and 15.55% were found to have impaired fasting glycemia during laboratory tests. In a feedback, the finding of NAFLD led to the diagnosis of prediabetes in these 23 patients. Our data regarding the frequency of DM among patients with NAFLD are largely similar to those presented in the world and Bulgarian literature, where most data indicate a frequency between 50-70%. In Tomova's group of NAFLD patients, 55.83% had proven type 2 DM. According to literature data, one-third to two-thirds of diabetics have NAFLD, and our study found that two-thirds of NAFLD patients have glycemic disorders – DM or prediabetes. The high incidence of NAFLD among diabetics makes them a target group for active search for steatosis. When NAFLD is established and there is no history of DM, we recommend assessment of glycemic status with an active search for underlying diabetes or prediabetes. Our analysis showed that there was a statistically significant relationship between the presence of DM and hepatic steatosis, but in our study group this relationship was weak with a correlation coefficient rho=0.209, p=0.056. According to literature data, DM is associated with a more aggressive course of the disease and a higher degree of fibrosis, which was confirmed by the results in the group we studied. A moderately strong correlation was found between the degree of fibrosis and the presence of type 2 DM (rho=0,388, p=0,001).

Another important component of the metabolic syndrome and a predictor of NAFLD is Hypertensive Heart Disease (HD). In the group studied by us with a history of proven HD, there are 120 out of a total of 148 patients, i.e. 81.08%. Only 28 patients (30.83%) had no history of proven hypertensive disease and no physical evidence of elevated blood preasure. The analysis shows that patients with II stage of HD predominate. Bulgarian authors indicate a similar but lower frequency of HD among examined patients with NAFLD – 74.1% according to Balabanska's data and 54% according to Tomova's data. According to literature data, 50% of patients with HD also have hepatic steatosis. Our analysis proved that there is a statistically significant positive relationship between the presence of HD and its degree relative to the degree of steatosis (rho=0.277, p=0.011). The degree of steatosis increases in parallel with the increase in the stage of HD and its duration. Examining the correlation between HD and fibrosis stage, we found similar data (rho=0.267, p=0.010). A statistically significant but weak relationship was found.

The most common and generally accepted risk factor for NAFLD is obesity. According to literature data, the risk of hepatic steatosis increases with the degree of obesity, and all degrees of obesity, including overweight, are risk factors for the occurrence of hepatic steatosis. Visceral type of obesity, which is considered to be the main risk factor for the occurrence of hepatic steatosis, is of particular importance for the occurrence of NAFLD. Visceral obesity is defined as having an abdominal circumference ≥94 cm for men and ≥80 cm for non-pregnant European women. Therefore, anthropometric studies - determination of

height, body weight, waist circumference and, accordingly, BMI in each patient with suspected NAFLD are of particular importance.

According to literature, up to 95% of overweight or obese people have NAFLD. In the group of patients studied by us, only 5.34% were of normal body weight, 25.19% were overweight, and all the rest were of various degrees of obesity, with no significant differences in the degrees of obesity between the two sexes. The correlation analysis performed proved a strong degree of association between the measured body mass index and the degree of steatosis (r=0.503, p=0.001). This testifies to the parallel increase in the degree of steatosis with the increase in body mass index values. Correlation analysis also demonstrated a moderately strong association between increasing BMI and increasing liver fibrosis (r=0.379, p=0.001). Therefore, patients with a higher BMI are more likely to have advanced fibrosis.

The pathogenetic relationship of the visceral type of obesity with the development of NAFLD has been proven. The easiest and most affordable way to prove visceral obesity is to measure waist circumference. Increased waist circumference is associated with visceral obesity. All participants in our study had an abdominal circumference above the WHO cut-off for acceptance of visceral obesity in Caucasians. The majority of patients in our study (88.46% of men and 98.65% of women) even had a second level of visceral obesity defining a significantly increased risk of metabolic-related diseases and an increased general health risk. Analysis of the results showed a strong correlation between the degree of steatosis and waist circumference (r=0.481, p=0.001). The analysis carried out found a strong correlation between the stage of fibrosis and the waist circumference, which proves that as the waist circumference increases, so do the liver density values, which is the main predictor of the progressive course of the disease (r=0.463, p=0.001). To measure the factorial influences and the specific effects of the influence of MS components on the degree of steatosis, we used regression analysis.

The regression model compiled by us found that the increase in waist circumference, which is also the main factor presenting the degree of visceral obesity, has the greatest predictive power for the appearance and degree of steatosis assessed with CAP (p=0.002). The regression model showed that a 1.6 cm increase in waistline resulted in a reciprocal increase in the degree of steatosis by 2 dB/m. All these data prove the importance of determining waist circumference and warrant its routine measurement in the clinical evaluation of patients with NAFLD.

The determination of anthropometric parameters of obesity and especially of visceral obesity are alarming for an underlying liver disease - NAFLD and could even be interpreted as predictors of a possibly more aggressive disease with a higher degree of steatosis and fibrosis.

Comprehensive clinical evaluation of patients with NAFLD includes, in addition to analysis of anamnestic data, data on comorbidities, risk factors, and physical data, as well as interpretation of laboratory data. There is no specific laboratory marker proving the presence of steatosis. Laboratory tests important for the clinical assessment of patients with NAFLD are the liver parameters - AST, ALT, GGT, AF, total bilirubin, lipid profile with examination of total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol. The examination of platelets and albumin is also important.

In the group of patients studied by us, there were 23.80% of patients with an increased value above the reference value for ALT, the same number of patients with an increase in AST - 23.80%, i.e. we observe a parallel increase in the values of both indicators. Patients with an increase in ALT up to two times above the norm (63%) predominate, and in the remaining 27% the increase is mostly up to three times above normal, and only three of them had a more significant increase. Increased values of GGT were found in 44% of the subjects. To a much lesser extent, an increase in AF was recorded, only in 10% of the studied patients, and the increase was mild up to a maximum of 2 times the norm. Similar results were obtained for total bilirubin, which was slightly elevated in only 8% of patients, mainly due to an indirect fraction. Therefore, in the highest proportion of patients, an increase in GGT values and, to a lesser extent, AF and bilirubin were found. This gives us reason to assume that GGT is mostly related to underlying steatosis and suggests the presence of a metabolic disorder. Similar data on the percentage increase in laboratory markers for hepatocytolysis and cholestasis were also found by other Bulgarian authors. Tomova found a corresponding percentage increase for ALT 52%, AST-48%, GGT-85%, AF-32.9% and total bilirubin - 3%. The data from Balabanska's study indicate the following frequencies of increase in ALT-45.3%, AST-37.6%, GGT-50.6%, AF-10.14%, bilirubin - 0%. Minimal biochemical liver laboratory tests should necessarily include ALT, AST and GGT. The study of AF, total and direct bilirubin give us information in differentiating cholestasis syndromes with another cause.

Very important laboratory data in the clinical evaluation of patients with NAFLD are lipid profile studies. Two of the components of the lipid profile – triglyceride level and HDL cholesterol are risk factors forming the metabolic syndrome and related to the genesis of NAFLD. Literature data indicate that 50% of patients with dyslipidemia develop NAFLD. In the group of patients studied by us, the number of those with registered deviations in the lipid profile is significant. An increase in the value of triglycerides was found in 47.48%, a decreased value of HDL cholesterol was found in 59.7% and an increased value of total cholesterol respectively in 56% of the studied patients. Lipid disorders are undoubtedly related to the occurrence of hepatic steatosis. Our correlation analysis showed a weak association between the degree of steatosis and the degree of increase in triglycerides ($r=0.010$, $p=0.929$), with no association of the triglyceride value with the stage of fibrosis. We assume that elevated triglycerides are relevant to the formation of steatosis and probably to some extent to the degree of steatosis, but not to the stage of fibrosis. In the correlation analysis of the increase in cytokeratin 18, as a marker of hepatocytolysis, a statistically significant correlation was found between the degree of increase in triglycerides and the degree of increase in CK18. Therefore, we believe that the increase in triglycerides is related to the inflammatory status in patients with NAFLD and, accordingly, the increase in Tg is related to the progressive course of the disease.

Ultrasonography is a cornerstone in the diagnosis of NAFLD. It gives us a qualitative and semi-quantitative assessment of hepatic steatosis. The criteria for ultrasonographic diagnosis of hepatic steatosis and assessing its degree are generally accepted. A detailed examination with a description of the findings relevant to the grading of steatosis is recommended. According to literature, steatosis is detected sonographically in 20% to 30% of steatotic hepatocytes. In our study, 45% of patients had severe steatosis, 34% had moderate

steatosis, and 19% had mild steatosis. Ultrasound examination with great reliability can confirm the presence of liver cirrhosis, even in the absence of decompensation. In 2 of the studied patients, cirrhosis was proven by echography, which was confirmed by the measurement of liver density by fibroscan.

The performed correlation analysis found a high degree of dependence between the degree of steatosis assessed by ultrasound and CAP from fibroscan (rho=0.462, p=0.001). An increase in CAP was found with each degree of steatosis. The statistical analysis performed using the One-way ANOVA method showed statistically significant differences between the three degrees of ultrasound-detected steatosis. We compared the mean values of the three degrees of steatosis by the subsequent Tukey's Post Hoc Test. It was found that there was a significant difference between first and second degree of sonographically determined steatosis (p=0.004) and between 1st and 3rd degree (p=0.001), but no significant difference was found between 2nd and 3rd sonographically determined degree (p=0.201). Therefore, for a more accurate determination of the established by ultrasound steatosis and the differentiation of moderate from severe steatosis, it is recommended to perform elastography with the determination of CAP. All these data prove the indisputable importance of ultrasound examination as a first-line imaging method for screening and proving NAFLD.

Another imaging method of great importance in the clinical evaluation of patients with NAFLD, entering into the wide clinical practice is transient elastography, enabling the staging of the degree of steatosis by assessing CAP and elastography to determine the stage of fibrosis.

Ultrasonography could serve to screen patients with NAFLD, and fibroscan enables the quantification of steatosis and fibrosis. To determine the degree of steatosis according to CAP, based on literature data from studies and meta-analyses around the world and in Bulgaria, we accepted the following limit values: S0 - at values below 215 dB/m, S1 - at values up to 253 dB/m, S2 - at values up to 300 dB/m and S3 for values above 300 dB/m. According to the accepted criteria, the largest number of patients in the group studied by us are with grade S3 steatosis - 53.58% of the total number, with grade S2, respectively, 32.14% and the smallest number are patients with grade S1 - 13 %. S0 steatosis was found in only one patient. In the group studied by us, the gender distribution of the different degrees of steatosis was almost the same. A high degree of correlation was found between the ultrasound examination and the determined steatosis from CAP, but a lower ability of the ultrasound examination to differentiate between 2 and 3 degrees of steatosis was demonstrated. Determination of CAP is a more accurate method for quantitative staging of the degree of steatosis. Quantification of steatosis by fibroscan CAP, on the other hand, allows monitoring and control of changes occurring during treatment of patients with NAFLD. Based on the results of the study of CAP in the patients in our study group, we performed a correlation and regression analysis with the components of the metabolic syndrome and established the dependencies described above, giving us the opportunity to draw up a plan for conducting screening in the patients with NAFLD.

For non-invasive determination of steatosis in clinical practice, several indices are used, calculated according to relevant formulas. For Fatty liver index (FLI), BMI, abdominal

circumference, Tg level and GGT are important, which according to the data obtained from our study are of great importance for the clinical evaluation of patients.

When calculating the HSI (Hepatic steatosis index), the values of ALT, AST, BMI, gender and the presence of DM are used, which as independent parameters are essential for the evaluation of patients with NAFLD. According to literature data, at relevant values, these indexes have a very good negative predictive value for rejection, and also a positive predictive value for proving steatosis.

Our correlation analysis found a moderately strong statistical relationship between CAP values and steatosis indexes (FLI and HSI), therefore CAP increased in parallel with FLI and HSI values.

The additionally performed One-way Anova analysis proved that both indexes can be used as screening for steatosis, and FLI could better guide us on the degree of steatosis as well. Steatosis indexes represent a simple and accurate method for predicting steatosis and are convenient for selecting patients for ultrasound examination and the need for consultation to change lifestyle. They can be used to predict liver morbidity.

According to recent literature data, fibrosis is unequivocally accepted as the main indicator determining the severity of liver damage, the stage of the disease and the possible progression of the disease to cirrhosis. Therefore, in the staging of patients with NAFLD, the determination of the stage of fibrosis is of particular importance. This can be specified by invasive biopsy examination with histology or by noninvasive fibrosis staging by imaging elastography techniques or serum biomarker scores. The invasiveness of the biopsy with even a small risk of complications, the variability and subjectivity in reading the result, the mosaicity of the changes in the liver and the possibility of "sample error" due to the small representativeness of the sample and, last but not least, the fear of the procedure and the refusal to perform it on the part of patients, the impossibility of screening and staging the disease among large groups of people, require in modern hepatology the use of non-invasive methods for clinical evaluation of the stage of fibrosis.

Fibroscan transient elastography measures liver density in kPa and thus assesses the stage of fibrosis. Results vary between 1.5 and 75 kPa. The threshold values for individual stages of fibrosis differ between individual nosological units – viral hepatitis, alcohol, NAFLD. Fibrosis grading from 0 to 4 was performed according to accepted cut-off values from meta-analyses of referrals in patients with NAFLD and NASH.

In our studied group of patients, those with absent or mild fibrosis predominated (F0 and F1, 66%), which is consistent with literature data on the characteristics and clinical course of the disease worldwide. Patients with advanced fibrosis and cirrhosis, which are the main predictors of an aggressive course of the disease, represent a significant proportion of the studied group (F3 and F4, 17.6%), which necessitates their active search and more active monitoring in view of the high frequency of liver-related morbidity and morbidity from cardiovascular diseases and the need for their treatment. The proportion of patients with significant F2 fibrosis is 16.4%, and these patients are in a reversible phase of liver changes and would benefit from dietary and exercise measures and treatment of metabolic disorders.

Therefore, assessment of liver density is of great importance for the clinical evaluation of patients, their risk stratification and determination of further behavior.

Using liver density results from fibroscan, we investigated the relationships between clinical parameters and the strength of their effects on fibrosis. A moderately strong association was found between liver density and body mass index, as well as a strong correlation with increasing waist circumference. The correlation between the stage of fibrosis and the presence of type 2 DM is moderately strong, while the correlation with the presence of HD is weak. This data also gives us rationale for developing a strategy for screening and intensive surveillance among target groups with a likely aggressive course of the disease, such as patients with high BMI, large abdominal circumference and type 2 DM.

Examining the correlation between the degree of fibrosis and the laboratory parameters AST, ALT, GGT, platelets and serum triglycerides, we found a statistically significant relationship only between the values of AST and fibrosis, and the correlation is weak, which proves the increase of AST in parallel with fibrosis.

Information about the stage of fibrosis is also provided by the serum biomarker scores. A number of studies have compared the three scores (FIB-4, NAFLD fibrosis score and APRI) assessing fibrosis. NAFLD fibrosis score and FIB-4 according to literature data, have the highest accuracy and negative predictive accuracy of over 90% to rule out fibrosis. According to the literature, fibrosis scores have very good diagnostic value for predicting advanced fibrosis, but do not distinguish mild from moderate fibrosis. We compared the results of the fibrosis scores obtained from our group of patients with the liver density results from fibroscan and found a statistically significant correlation for all three scores. The highest correlation was found for NAFLD fibrosis score ($r=0.432$, $p=0.001$), followed by FIB-4 ($r=0.365$, $p=0.001$) and APRI score ($r=0.327$, $p=0.002$). One-way ANOVA analysis performed to determine statistically significant differences between the four groups of fibrosis determined by mean values of liver density by fibroscan found statistically significant differences for NAFLD fibrosis score ($F=3.562$, $p=0.011$) and for FIB-4 ($F =2.812$, $p=0.030$). For APRI only, the comparison between fibrosis groups was not statistically significant ($F=2.193$, $p=0.77$). The performed Post Hoc Test according to the Tukey method showed that in the group of patients studied by us, statistically significant differences were found between the absent and mild degrees of fibrosis, compared to F3 and F4. This proves, similar to the literature data, that fibrotic scores can certainly be used as a first-line risk stratification to rule out advanced disease.

In our study, we performed a comparative analysis between the increasing of steatosis and increasing of fibrosis stage. We found a correlation of higher CAP value with higher F value ($F=5.270$, $p=0.001$). A detailed examination by fibrosis groups revealed that only in F4 versus F3 ($p=0.965$), the difference was not significant between the CAP values in the advanced fibrosis groups, which was explained by the similar high CAP value in both groups.

We performed a detailed comparative analysis between the patients without fibrosis and the four fibrosis groups defined by fibroscan versus BMI, abdominal circumference in centimeters, ALT and AST. Descriptive analysis by group found a lower mean BMI in patients with absent fibrosis and mild fibrosis compared to F3 and F4. There was also an increase in abdominal circumference with increasing degree of fibrosis and higher mean AST values for

F3 and F4, which had mean values above the mean relative to no and mild fibrosis. By ANOVA analysis, it was found that there was a statistically significant difference between the four fibrosis grades in terms of abdominal circumference only (F=5.917, p=0.000), which proved its significance as a predictor of advanced fibrosis. The analysis did not show statistical significance for BMI and AST, which is explained by the fact that the majority of our studied patients were in the group with absent or no significant fibrosis (66%). Statistical significance in difference of ALT values by fibrosis subgroups was not found.

Nonalcoholic steatohepatitis is considered to be the progressive form of NAFLD, leading to the development of fibrosis and cirrhosis, and is therefore associated with a greater likelihood of liver complications. The reliable method to prove inflammation is biopsy, the negatives of which we have already considered. Multiple serum biomarkers have been investigated for NASH, but the only independently validated such biomarker is serum cytokeratin 18. As a marker of hepatocyte apoptosis, it is characteristic of steatohepatitis. According to literature, CK 18 levels vary and are significantly higher than in healthy controls, correlate with disease severity, and are much higher in patients with steatohepatitis than in those with simple non-progressive steatosis. Experience with the use of CK 18 in routine practice is still lacking, and there is variability in the cut-off values and their respective diagnostic accuracy among different studies. There is a lack of a large study among the Bulgarian population on the relationship between NAFLD and CK 18. There is no accepted reference limit of the norm for serum cytokeratin 18 in ng/ml for the Bulgarian population, therefore we adopted the value proposed by the manufacturer. The measured mean value for CK 18 in our patient group was 3.01±10.42 ng/ml, with a minimum value of 0.1 ng/ml and a maximum value of >80 ng/ml. In the group studied by us, normal values were found in 90% of patients, and an increase which is a mark for of steatohepatitis was found in 10% of patients. Visceral obesity, with abdominal circumference corresponding to a significantly increased risk, insulin resistance with HOMA-index above 2.5 and evidence of metabolic syndrome were also present in all patients with elevated CK 18.

The correlation analysis used to examine the relationship between clinical parameters and establish the strength of their influence on cytokeratin values showed a moderately strong positive relationship between the level of triglycerides and SC18 (r=0.318, p=0.012), indicating an increase in the value of triglycerides in parallel with the values of CK18. The relationship between the increase of Tg and the inflammatory status determined by the level of cytokeratin 18 leads us to the conclusion that the increased level of triglycerides is associated with a high probability with inflammatory processes. No correlation was found regarding the increase in BMI and abdominal circumference on the one hand and the increase in CK 18 on the other. Correlation analysis did not give significant results for the relationship between an increase in the value of cytokeratin 18 and the presence of HD, DM and MS.

The lack of correlation by the Pearson method with the laboratory parameters AST, ALT, GGT and CRP we considered to be due to the lack of a large variation in the values of cytokeratin 18 in our studied group of patients (n=61). The Independent Samples T-test used, by which we compared the mean values of ALT, AST, GGT and CRP between patients with normal and elevated CK 18, showed significantly increased values of ALT, AST and GGT in the group with elevated CK 18 compared to that with normal, as the differences found were

statistically significant (t=-2.958, p=0.004; t=-3.729, p=0.001; t=-2.266, p=0.027, respectively). Only statistically significant relationships were not found between CRP and CK 18 values. The above results give us reason to assume that the detection of elevated CK 18 is a reliable method to prove inflammation in NAFLD. We believe that, in order to increase the reliability of the results, it is necessary to conduct the study among a larger group of patients, and it is also necessary to validate reference limits for CK 18 among the Bulgarian population.

According to literature data, an increase in CK 18 correlates with a higher degree of fibrosis. In our study, the correlation analysis between the level of CK 18 and the degree of steatosis and the stage of fibrosis did not find a statistically significant relationship. These data are explained by the fact that in the studied group few patients have advanced fibrosis, determined according to the value of the fibrosis scores. Patients with absent or mild fibrosis predominate. A larger sample of patients with different liver densities is likely needed to conclude whether elevated CK 18 levels correlate with higher-grade fibrosis. Further studies are necessary to confirm the role of CK 18 as a marker of hepatocyte inflammation in routine practice.

6. Conclusion

Nonalcoholic fatty liver disease is the phenotypic hepatic manifestation of the metabolic syndrome. The role of the components of the metabolic syndrome in the occurrence of hepatic steatosis and its progression is indisputable and clarified. This relationship is bidirectional. The presence of hepatic steatosis is considered to be a predictor of the occurrence of cardiovascular diseases, carcinomas of different localization and increased mortality not only from liver-related causes, but also from various causes. 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, and 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 to monitor disease progression and therapeutic response. Liver biopsy with histological examination, although the gold standard for the diagnosis of steatohepatitis and for the staging of fibrosis, does not meet these criteria given its invasiveness, inability to screen and follow-up. Such easily accesible, safe, reliable and accurate methods enabling the quantitative assessment and monitoring the degrees of steatosis and the stage of fibrosis are the non-invasive serum biomarker scores and imaging studies. They are increasingly entering the wide practice and proving their importance. Nowadays, we can safely say that these tests in most cases replace histological examination for the diagnosis of NAFLD and its staging.

The diagnosis of NAFLD/NASH is complex and requires a combination of different methods, starting from history and physical examination, including anthropometric parameters (height, weight, BMI and abdominal circumference), biochemical laboratory parameters, imaging studies and morphological examination. The established score systems for steatosis and fibrosis assessment based on serum biomarkers and clinical characteristics

of patients are an easily accessible and reliable way to quickly orientate on the presence of steatosis and the stage of fibrosis. Echographic examination is also an affordable and safe method for screening, establishing steatosis and its semi-quantitative grading. The use of transient elastography is unquestionably imposed and confirmed in modern clinical practice, as one of the main reliable and harmless diagnostic methods enabling an accurate assessment of the degree of steatosis through CAP and at the same time determining the stage of fibrosis. Given its ease of implementation, harmlessness and the possibility of multiple repeatability, this method can be used to follow the course of the disease and monitor the therapeutic response to lifestyle modification and eventual drug treatment. Transient elastography enables more precise screening and identification of patients at high risk of an aggressive disease outcome, i.e. detection of patients with higher-grade fibrosis.

Screening for NAFLD/NASH is recommended in patients with metabolic syndrome and patients with type 2 DM in whom a more aggressive course of the disease is suspected or in patients with abdominal obesity, which is a major predictor of high-grade steatosis and fibrosis. The end result of this would be timely diagnosis, treatment and slowing of disease progression.

The increase in knowledge about non-alcoholic fatty liver disease and the clinical focus on it leads to timely diagnosis of the disease in the early stages, with the aim of influencing and the possibility of reverse development of liver changes. Knowing the features of the disease and its progressive course makes it possible to make an early diagnosis and prevent the progression of the disease.

7. Findings

1. Adequate clinical evaluation of patients with NAFLD is of great importance given the diverse forms and stages of the disease and should include a full assessment of accompanying metabolic abnormalities, respectively the components of the metabolic syndrome.

2. The presence of type 2 DM is associated with a risk of developing higher-grade fibrosis in NAFLD patients and a more aggressive course of the disease. DM suggests NAFLD, but the presence of steatosis also suggests DM. An active search for glycemic disorders among patients with NAFLD is recommended.

3. Visceral obesity is a major risk factor for NAFLD. The degree of steatosis correlates to the greatest extent with the increase in BMI and above all the abdominal circumference. Increased abdominal circumference is the main predictor of the occurrence and severity of steatosis and also suggests a higher degree of fibrosis. Routine measurement of BMI and abdominal circumference is recommended in patients with established NAFLD.

4. Ultrasonography is a first-line imaging modality suitable for screening and grading NAFLD.

5. Determination of CAP by vibrational elastography (fibroscan) is an accurate non-invasive method for the quantitative staging of steatosis and is recommended for routine use in the clinical evaluation of patients with NAFLD.

6. The steatosis scores - FLI and HSI represent a simple and accurate method for predicting the presence of steatosis and are convenient for selecting patients for ultrasound examination. They can be used as a screening for steatosis, but also show a good correlation with degrees of steatosis.

7. Determination of liver density by transient elastography is an accurate and reliable method for staging fibrosis in patients with NAFLD and is of utmost importance for the clinical evaluation of patients, and their risk stratification and determination of further treatment and follow-up behavior.

8. Fibrosis scores (NAFLD fibrosis score, FIB-4, APRI) showed a statistically significant correlation with fibrosis values determined by fibroscan. Fibrosis scores are a reliable tooles of ruling out advanced fibrosis.

9. The examination and determination of elevated cytokeratin 18 values is a reliable method of proving inflammation in NAFLD.

8. Contributions

Contributions of a scientific - applied and confirmatory nature

1. A complete clinical evaluation of NAFLD patients was performed, confirming the importance of metabolic risk factors for disease progression.

2. The role of anthropometric parameters in the clinical evaluation of patients with NAFLD has been confirmed.

3. The role of ultrasonography for the diagnosis and screening of NAFLD in daily practice has been confirmed.

4. The importance of determining the degree of steatosis by CAP as a routine imaging method in the clinical evaluation of patients with NAFLD has been demonstrated.

5. The importance of measuring liver fibrosis by transient elastography has been confirmed and its use in routine practice for staging liver fibrosis in NAFLD and identifying patients at high risk of progressive disease has been validated.

6. A comparative analysis was made between non-invasive serum and imaging methods for staging steatosis and fibrosis and their role in daily clinical practice was explained.

Contributions of original character

1. An algorithm for screening and diagnosis of NAFLD patients is proposed.

2. For the first time, a study of cytokeratin 18, as a marker of inflammation, was performed in a wide population of patients with NAFLD in Bulgaria.

9. List of publications and participation in scientific forums related to the topic of the dissertation work

1. Boykova P, NON-INVASIVE BIOMARKERS FOR ASSESSMENT OF NON ALCOHOLIC FATTY LIVER DISEASE, European bridging meeting in gastroenterology, 15th – 16th November 2020, Warsaw, Poland

2. Boykova P., Ivanova I, Kotsev I, Non-invasive biomarkers for the assessment of Non-Alcoholic Fatty Liver Disease (NAFLD), Bulletin of the Union of Scientists Varna, 2019, pp. 10-15;

3. Boykova P., Gancheva D., Clinical case of a patient with liver cirrhosis in non-alcoholic fatty liver disease and pregnancy, Bulletin of the Union of Scientists Varna, 2019, volume 24, pages 5-9;

4. Boykova P. Non-alcoholic fatty liver disease (NAFLD) – a growing health problem in Bulgaria, GP News, issue 4 (234), April 2020; 30-34

5. Boykova P. How to treat non-alcoholic fatty liver disease today?, GP News, issue 4 (263), 2022; 14-18

6. Can we protect ourselves from NAFLD?, Gastroforum Varna, November 2018 - prevention; Oral report

7. Modern diagnosis and treatment of NAFLD, Gastroforum Varna, November 2019 – news; Oral report

8. Non-alcoholic fatty liver disease - news, Gastroforum Varna December 2020 - news; Oral report

Thanks to everyone who contributed to this work:

My supervisor, Prof. Dr. I. Kotsev, MD, for his guidance and help.

Assoc. Dr. Irina Ivanova, MD, Head of Gastroenterology, Hepatology and Nutrition Clinic at UMBAL "St. Marina" - city of Varna, for the help and friendship.

Prof. Dr. Yana Bocheva, MD head of the Department of Clinical Laboratory at UMBAL "St. Marina" - city of Varna, for the help and expert, precise work.

Colleagues from the Gastroenterology, Hepatology and Nutrition Clinic at St. Marina" - the city of Varna for the cooperation, the courage they inspired me and the pleasure of working together.

Colleagues from the Clinical Laboratory at UMBAL "St. Marina" - city of Varna.

Associate Professor Dr. Silvia Nikolova, Ph.D. for the expert statistical processing of the data.

My mother for her support, belief in me and courage.

My family - my husband and my wonderful daughters Ilina, Svilena and Monika for their endless love and support.