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# NON-INVASIVE ALGORITHM FOR RISK STRATIFICATION IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER (NAFLD)

**PHD THESIS SUMMARY**

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## General part

### 1. Introduction

In recent decades it has become increasingly evident that non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are leading causes of liver disease in Western countries. More recent data confirm that NAFLD and NASH also play a very important role in the Middle East, Far East, Africa, Caribbean and Latin America [1].

Non-alcoholic fatty liver represents a wide spectrum of liver disorders characterized histologically by macrovesicular hepatic steatosis in more than 5% of hepatocytes, in the absence of alcohol consumption (>10 grams/day, in the case of women and >20 grams/ day in the case of men) or secondary causes of steatosis. [2]

The prevalence of simple hepatic steatosis is highly variable, depending on the population studied, and is considered a manifestation of the metabolic syndrome. Recent data have reported differences in the prevalence of NAFLD between racial and ethnic groups. [3]

Non-alcoholic fatty liver disease is a condition found in patients who deny the consumption of alcoholic beverages, which includes a wide spectrum of liver damage: simple steatosis defined by the accumulation of triglycerides inside the hepatocytes and non-alcoholic steatohepatitis which involves lobular liver inflammation, ballooning of hepatocytes, fibrosis with potential evolution towards cirrhosis, hepatocellular carcinoma up to terminal liver failure. [4]

#### 1.1 Natural history

Nonalcoholic fatty liver disease (NAFLD), which was previously known by this name, represents a group of liver diseases that are not caused by alcohol consumption, viral infections, autoimmune conditions, drug administration, or genetic factors. Recently, the name of this condition has been changed to "Fatty Liver Associated with Metabolic Dysfunctions" (MAFLD). [5] This new nomenclature correctly indicates the determinants of the disease, namely the close association with metabolic disorders, and avoids the old term (ie, nonalcoholic). [5]

Most patients with NAFLD have isolated steatosis, with good prognosis and minimal risk of progression to cirrhosis (<4%). Patients with NASH (~20-25%) have an increased risk of progression to fibrosis and cirrhosis (20%). Recently, investigators have included, alongside simple steatosis and steatohepatitis, a new entity, steatosis with minimal inflammation, insufficient for the diagnosis

steatohepatitis. The proportion of patients with steatosis and minimal inflammation is not precisely known, but it is clear that these patients require periodic surveillance and follow-up.

The prevalence of NAFLD is increasing rapidly worldwide, in parallel with the increase in obesity and type 2 diabetes. The prevalence of NAFLD in the general population is estimated to be 20–30% in Western countries and 15% in Asian countries. In Saudi Arabia, the prevalence of NAFLD, assessed by computed tomography, is approximately 10%. [6]

**2. Clinical and paraclinical diagnosis**

Non-alcoholic fatty liver disease is associated with central obesity, insulin resistance, metabolic syndrome and drug toxicity. [7] All these things can be emphasized by a thorough anamnesis. In order to diagnose possible chronic liver diseases and finally to have the opportunity for adequate treatment, a battery of laboratory tests must be performed, such as the determination of AgHbs and AgHbe antigens, but also AcVHC - to exclude liver viruses, for Willson's disease - ceruloplasmin dosage, to exclude hemochromatosis - alpha1 antitrypsin and transferrin dosage, and to exclude some autoimmune pathologies, we need to dose a set of specific antibodies: antinuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA-represents a serological marker for autoimmune hepatitis type 1 and biliary cirrhosis primitiva) and LKM- antibodies represent a serological marker for autoimmune hepatitis type 2, the most common form of juvenile autoimmune hepatitis. Imaging evaluations are also important, standard ultrasonography and Doppler of the liver and of the portal venous system, possibly completed with a superior imaging investigation such as computed tomography and nuclear magnetic resonance. [8]

The common causes of steatosis are divided into 2 subtypes depending on the degree of steatosis. Macrovesicular steatosis has as frequent causes excessive alcohol consumption, chronic hepatitis with the C virus, Wilson's disease, parenteral nutrition, lipodystrophy, but also the consumption of certain drugs such as: amiodarone, methotrexate, tamoxifen and corticosteroids. Microvesicular steatosis is most often found in patients with Reye's syndrome, acute fatty liver of pregnancy, Help syndrome, congenital genetic anomalies (Wolman's disease, stratified cholesterol storage disease) but also the consumption of drugs - Valproate and anti-retrovirals. [9] [10, 11].

Metabolic risk factors for the development of NASH include: obesity, type 2 diabetes, insulin resistance, prolonged fasting, rapid weight loss and hypertriglyceridemia, as well as hypercholesterolemia. [12]

Non-alcoholic fatty liver disease is the most common cause of chronic liver disease worldwide. NAFLD is considered a metabolic condition determined by the interaction between genetic, hormonal and nutritional factors, with oxidative stress, inflammation, lipotoxicity and peroxidation, as well as mitochondrial dysfunction, being particularly recognized as being involved. [13]

Usually, most patients are asymptomatic. Some patients may experience progressive fatigue or slight discomfort in the right hypochondrium. It is believed to occur as a result of distension of the liver capsule. [14]

From the point of view of personal pathological antecedents, we are particularly interested in operations performed at the gastric level (eg: jejuno-ileal bypass). [15] There are also a number of drugs that are associated with non-alcoholic fatty liver disease.

Other important factors associated with insulin resistance, which seem to be related to the association of NAFLD but which have not been officially reported, are: sleep apnea syndrome, hypothalamic-pituitary disorders, psychiatric disorders, irregular periods, infertility and/or hirsutism , polycystic ovary syndrome (PCOS). [16] Another frequent association, with a particular clinical significance, is with colonic polyps and colorectal carcinoma (CRC); NAFLD is associated with the presence of large numbers of polyps, polyps with a high malignancy potential, with an increased rate of CRC occurrence and with an unfavorable response to CRC treatment. [17] Last but not least, obese and diabetic patients, and implicitly patients with NAFLD, have an increased rate of gallstone development. [18]

Heredo-collateral antecedents also play an important role, especially those related to diabetes and the association with non-alcoholic fatty liver disease.

It is recognized that, frequently, patients diagnosed with NAFLD do not present any symptoms attributable to liver disease, but at the same time, a diverse range of debilitating symptoms is present, which appear to be unrelated to the underlying liver changes. Some of these symptoms can be attributed to conditions associated with NAFLD, particularly the presence of diabetes and cardiovascular disease. An important contributor is obesity and sleep apnea syndrome (OSA), both of which are very often associated with NAFLD. As previously discussed,

NAFLD is often diagnosed in those patients with non-specific symptoms in the context of changes in laboratory tests. These symptoms include fatigability, right hypochondrial abdominal discomfort, and daytime sleepiness, but the symptom profile is likely to expand as our understanding of the presentation of patients with NAFLD improves.

The diagnosis of NAFLD is suggested by the evidence, sometimes, of hepatomegaly during the physical examination or by the detection, in overweight people or those who present elements of the metabolic syndrome (hyperglycemia, dyslipidemia, hypertension), of some changes of liver function tests, unexplained by the excessive consumption of alcoholic beverages or the presence of other liver disorders, and/or the finding of increased echogenicity of the liver or other features of fatty liver, during a routine ultrasonographic examination. [19]

The physical examination follows the anthropometric assessment (body mass index), but also the detection of the stigmata of a chronic liver disease such as: jaundice, collateral circulation, muscle atrophy, the characteristics of the liver (size, lower edge, surface, consistency), but also the presence of a possible splenomegaly and/or the presence of ascites fluid. [20]

The clinical examination of the liver reveals, in approximately 50% of cases, hepatomegaly with slightly increased consistency, with a rounded lower edge, regular, painless surface. If the disease is diagnosed at an advanced stage, skin signs of cirrhosis and signs of portal hypertension can be detected. [21]

The diagnosis of non-alcoholic fatty liver can be expressed later after excluding other causes of liver diseases and largely depends on the sincerity of the patient in his statement regarding the consumption of alcoholic beverages. No laboratory test clearly indicates the presence of steatosis or steatohepatitis. Increased serum values of the aminotransferases alanine aminotransferase (ALT) and aspartate aminotransferase (ALT) are apparently the only biochemical indicators of NAFLD. It is important to note that transaminase values do not fully correlate with histological activity, and that enzymes may have normal values despite advanced liver disease. While aminotransferases are the only liver enzymes affected, occasionally slightly elevated values of alkaline phosphatase (FA) can also be found. Except in cases of advanced disease, bilirubin, albumin, and coagulation factors are within normal limits. [22]

Using ultrasonography as a reference standard, FLI is an excellent diagnostic method, showing an accuracy of 84% in detecting fatty liver. Moreover, it has been shown that scores

high levels of FLI are associated with increased risk of type II DM, coronary events and

early carotid atherosclerosis.

The SteatoTest includes a series of biochemical markers that include: age, sex, body mass index (BMI), ALT, total bilirubin, gamma glutamyl-transpeptidase, cholesterol, triglycerides, blood sugar, □2- macroglobulin, apolipoprotein A1, haptoglobin - these predict steatosis with a sensitivity and specificity of 90%. [23] [23]

*The NASH test* should only be performed if the Steato-test is positive.

Regarding the serum markers of fibrosis, we mention the AST/ALT ratio and the APRI test, calculated according to the formula ASTx 100/no. Platelets x 10 9/l. [24]

The FibroTest can currently be used to test the necroinflammatory activity, as well as fibrosis, in non-alcoholic fatty liver. This test is made up of 5 markers: □2- macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and GGT. [25]

FibroMax includes a battery of serum markers used for the predictive diagnosis of liver fibrosis, which contains 5 tests: Steato-test, ActiTest- determines the degree of inflammation and hepatocytic necrosis, NASH Test for the diagnosis of non-alcoholic steatohepatitis, ASH Test- for the diagnosis of severe alcoholic hepatitis, the FibroTest that predicts the degree of fibrosis. Fibromax uses the values of 10 parameters depending on age, weight, sex. Height, and ALT, total bilirubin, gamma glutamyl-transpeptidase, cholesterol, triglycerides, blood sugar, □2-macroglobulin, apolipoprotein A1, haptoglobin and basal blood sugar.

Fib-4 is a score used to assess the stage of liver fibrosis, based on the measurement of serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and the presence or absence of hepatitis C virus infection. This score can be used in the evaluation of non - invasive liver fibrosis and can help determine the stage of liver disease and establish the appropriate treatment plan. The Fib-4 score is calculated using the mathematical formula that combines the serum AST and ALT values with the patient's age and blood platelet (platelet) level and can be interpreted according to pre-set critical values. [26]

The NAFLD Fibrosis Test is a non-invasive test used to assess the degree of liver fibrosis in patients with non-alcoholic fatty liver disease (NAFLD). This test is based on the level of four biomarkers: age, AST (aspartate aminotransferase), ALT (alanine aminotransferase) and albumin, which are analyzed together with individual risk factors such as body mass index (BMI), the presence of diabetes or hypertension.

*HOMA-1R*- first described in 1985 by Matthews and colleagues, the HOMA index is based on the

hypothesis that the relationship between basal blood glucose and basal insulinemia indicates the balance between insulin production by the pancreas and glucose production by the liver. This method can be applied regardless of age, gender or ethnicity.

Imaging methods have the ability to confirm steatosis, but also help to monitor the evolution of steatosis and can detect aggravating aspects of NAFLD, fibrosis - mainly through elastographic techniques and cirrhosis, as well as their complications, such as portal hypertension or hepatocellular carcinoma. Standard abdominal ultrasound, computed tomography, nuclear magnetic resonance, and MRI spectroscopy are used as imaging methods for the diagnosis of hepatic steatosis. These methods cannot differentiate steatosis from steatohepatitis. Imaging methods have an important role in the diagnosis and evaluation of patients with NAFLD.

A special imaging technique is transient elastography, which allows the quantification of steatosis through the controlled attenuation parameter (CAP). Superior methods are magnetic spectroscopy or proton density of the lipid fraction, excellent ways to quantify steatosis used in clinical studies. Computed tomography is an imaging method that detects fatty infiltration of the liver by the low attenuation of its image compared to that of the spleen or the paraspinal region. The sensitivity of this method in detecting NAFLD is 90%.[27] Magnetic resonance imaging is the most sensitive method, it can quantify steatosis, but it is the most expensive. These imaging techniques cannot differentiate steatosis from steatohepatitis and also cannot specify the stage of the disease (the degree of inflammation or the presence of liver fibrosis). [28]

Ultrasonography (US) is the main way to diagnose NAFLD and is the most widely used technique worldwide both in terms of availability and characteristics, being non-invasive and without the risk of radiation. Normal liver parenchyma has a homogeneous echostructure, similar to that of the kidney and spleen. The intracellular accumulation of lipids causes an increase in the echogenicity of the liver parenchyma, which is visualized as hyperechoic ("bright liver").

Elastographic ultrasonography (transient) is a sensitive method for the staging of liver fibrosis and with its help, the stiffness of the liver tissue can be determined, knowing that the stiffness of the liver is correlated with the degree of fibrosis. The machine for this exploration is called FibroScan. [29]

It represents an imaging technique used in assessing the elasticity of the liver tissue and can be used in the characterization of steatosis through (CAP) and the staging of liver fibrosis.

Impulse elastography - the principle of the method for quantifying liver fibrosis in patients with chronic liver diseases consists in integrating an ultrasound probe into the shaft of a vibrating device (Fibroscan, Echosens, France). This device emits vibrations of medium amplitude and low frequency (50Hz), which propagate in the form of a wave inside the liver. Velocity is measured, which is directly proportional to the deformability of the tissue, so in relation to its hardness. [30]

The greater the stiffness of the liver tissue, the faster the wave will propagate. The method allows the assessment of both fibrosis and hepatic steatosis.

Real-time ultrasonographic elastography (RTE-US) allows the detection of the mechanical behavior of tissues through an analysis of the ultrasonographic signals reflected to the probe while the tissues are compressed and decompressed, thus providing information complementary to that obtained by conventional ultrasonography. The distribution of tissue elasticity is calculated in real time and the result of the examination is displayed as a color-coded, transparent image superimposed on the gray-scale image of the examined structures. A recent approach to the interpretation of elastographic examinations, which eliminates human subjectivity, has been achieved by dynamic analysis using color histograms (computerized post-processing analysis) for each color frame of an elastographic examination.

## 2.1 HISTOLOGICAL DIAGNOSIS OF NON-ALCOHOLIC FATTY LIVER

In chronic inflammatory liver diseases, liver biopsy is still the gold standard for the diagnosis of liver lesions. Complications of liver biopsy are not frequent but can be dramatic. The reported mortality is about 1 case in 1000 liver biopsies, and major complications occur in about 0.3%.

Liver biopsy remains the standard method for confirming the diagnosis of NASH, staging fibrosis, establishing the degree of activity, and assessing response

to treatment. The specific histological features of steatohepatitis are: hepatocellular steatosis, ballooning degeneration of hepatocytes, mixed inflammatory infiltrate, hepatocytic necrosis, glycogenated nuclei and Mallory bodies. The main limitation is collection (sampling) error, which can lead to both underestimation and overestimation of disease severity. It is also an expensive investigation, requires expertise for interpretation, and has a low but potential risk of morbidity and even mortality. Liver biopsy can provide prognostic information and, above all, can exclude other concomitant liver diseases. [31]

Liver biopsy can be the source of errors due to the small size of the sampled fragment, or inappropriate dimensions of the puncture needle.

Complications can be minor - pain, vagal reactions, arterial hypotension, or major. Pain after percutaneous liver biopsy occurs in 25% of patients, is transient, has a low intensity and is felt as discomfort at the puncture site, sometimes it radiates to the right shoulder and requires mild analgesia. If the pain is of increased intensity, the occurrence of major complications, such as subcapsular hematoma, intrahepatic hematoma, hemobilia or intraperitoneal hemorrhage, must be taken into account. After the biopsy, small subcapsular or intrahepatic hematomas may appear - usually the patients are asymptomatic. [32]

Intraperitoneal hemorrhage is the most serious hemorrhagic complication of liver biopsy and occurs in the first 2-3 hours after the procedure.

## 2. NAFLD MANAGEMENT

Weight loss remains the standard treatment of NAFLD in the presence of overweight or obesity, but the optimal dietary treatment is unknown. Severe calorie and CHO restriction have been reported to result in almost complete disappearance of hepatic triglycerides within two weeks, but such programs cannot be sustained long term. [33] In obese patients with moderate type 2 diabetes, the hypocaloric, very low fat (3%) diet resulted in a 10% weight loss, which resulted in an 80% decrease in liver lipids. [34]

All patients should be advised that proper lifestyle and healthy diet improve prognosis, reduce steatosis and prevent progression of liver disease. Recommendations should be tailored to individual preferences and should include a reduction in saturated fat and fat intake

trans, higher fiber and omega-3 fatty acids and reduced intake of refined sugar, CHO, soft drinks and fruit juices.

A proper diet should always be paired with regular daily exercise. The ultimate goal is weight loss in overweight or obese patients with NAFLD.

### 3.1 Medication treatment

Metformin is an oral biguanide whose molecular mechanism is not fully elucidated, despite extensive clinical experience in its use. Biguanides, and implicitly Metformin, represent the therapy of choice used in the case of obese patients with diabetes; the glucoregulatory action taking place, first of all, at the level of the liver, by reducing the production of glucose, and secondly, at the level of the peripheral tissues, where it favors the uptake of glucose. [35]

Thiazolidinediones (TZDs) represent a category of antidiabetic drugs whose potential role in the treatment of NASH is still being evaluated. [35]

Ursodeoxycholic acid (UDCA) has many attractive putative mechanisms of action that have prompted its testing as a potential therapy for NAFLD. In addition to modifying the bile acid pool, UDCA has choleretic, anti-inflammatory, and anti-apoptotic effects and may also modulate the immune system response and mitochondrial integrity.[234] Previous studies have reported improvement of ALT and steatosis in patients with NAFLD and UDCA at a daily dose of 12–15 mg/kg alone or when combined with vitamin E.[35]

Bariatric surgery is considered to be the best therapeutic modality for patients with morbid obesity or associated comorbidities, such as sleep apnea syndrome, type II diabetes and hypertension[36]

Liver transplantation - is necessary in case of liver failure. 30-40% of patients with cirrhosis associated with NASH require liver transplantation. Unfortunately, non-alcoholic steatohepatitis can also occur post-transplant.[37,]

## 4. Prognosis

Several studies consider simple steatosis to be a benign condition. Progression to cirrhosis is very rare, approximately 3%. Whatever the form of the disease, the initial mechanism is the same, insulin resistance, while the different histological aspects are the consequence of secondary events.[38]

Regarding the potential for progression, NAFLD patients fall into two broad categories: NASH and non-NASH. The non-NASH subtype of NAFLD includes all patients with steatosis simple, as well as patients with steatosis and non-specific changes. Although NASH has a high potential to develop liver disease, the non-NASH subtype does not progress, or progresses very slowly. [39]

## Special part

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a liver disease associated with the metabolic syndrome and includes several types of clinical-pathological conditions: non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. This disease is characterized by the accumulation of triglycerides in the liver, in a proportion greater than 5%, and by excluding other causes of hepatic steatosis. NAFLD has become an important cause of liver disease-related mortality. The estimated prevalence of this condition has reached pandemic proportions, affecting approximately one-third of the general population. [1].

Correct diagnosis of NASH involves liver biopsy, the only procedure that can differentiate between NAFLD and NASH [40]. Liver biopsy continues to be the gold standard for diagnosis, but this investigation is an invasive procedure with significant risks, including mortality. The procedure is also subject to a number of errors, including sampling and interpretation errors, as well as interobserver variability [1].

Because of the risk of staging and diagnostic errors in a significant number of patients, due to histologic confirmation bias, and because liver biopsy is an invasive method that can involve various complications, there is ongoing research into the potential effectiveness of non-invasive tests in identifying individuals at increased risk of disease progression. Many patients refuse to undergo liver biopsy because of its invasive nature and potential complications. In this context, non-invasive alternatives are being explored that can provide relevant information to identify patients at high risk of disease progression.

A central aspect in the pathophysiology of NASH is the alteration of hepatic beta-oxidation. Fat accumulation in the liver is observed, along with an increase in hepatic uptake and free fatty acid (FAL) synthesis, which is offset by increased mitochondrial beta-oxidation and ketogenesis [40]. In light of these findings, non-invasive assessment of hepatic fatty acid metabolism could provide more accurate information on the progression from steatosis to steatohepatitis and could be useful in monitoring treatment efficacy.

Forty years ago, 13C-based breath tests were applied for the non-invasive investigation of liver mitochondrial function [41] A non-invasive test with good practicability in the investigation of liver functions can be considered the 13C- octanoic acid breath test. Octanoic acid is a medium chain fatty acid consisting of eight carbon atoms, and sodium octanoate is the sodium salt of this acid. Originally, octanoic acid was validated for use in a non-invasive breath test to assess gastric emptying rate of solids [41]. Octanoic acid has the advantage of being rapidly absorbed in the small intestine and transported via the portal vein to the liver, where it is metabolized by beta-oxidation to acetyl-CoA and CO2 [302]. The resulting CO2 is subsequently exhaled and can be collected in breath samples at various time intervals. This allows monitoring and quantification of CO2 release over time, providing information on octanoic acid metabolism and associated liver function.

## 2. Working hypothesis and general objectives

The arguments presented above were the basis of my study, which makes up the current doctoral thesis, which we will detail in the following chapters.

During the doctorate period, in which I carried out my work in the Bucharest University Emergency Hospital, a growing pathology we were dealing with was non-alcoholic fatty liver disease. Considering the global prevalence of NAFL, my interest has increased for this pathology and I have the right to research and follow this study directive, to describe liver damage and evaluate liver fibrosis through new non-invasive strategies to allows assessment of the full spectrum of NAFLD. In this context, the study **"Non-invasive algorithm for risk stratification in patients with non-alcoholic fatty liver"** was born.

**2.1. The aim of the study** was to develop new non-invasive strategies to allow the evaluation of the entire spectrum of NAFLD.

**2.2. The objective of our study** was to evaluate the effectiveness of the 13C-Octanoate breath test (OBT), as a surrogate marker of mitochondrial function, in differentiating patients with NASH from patients with simple steatosis (NAFL).

## 3. Research methodology

The study has an analytical, prospective design, in which patients evaluated in the Gastroenterology Department of the Bucharest University Emergency Hospital were included. The study was approved by the local Ethics Committee of the Bucharest University Emergency Hospital (no. 22052/ May 11, 2015), and all patients included in the study signed an informed consent and followed a protocol.

### 3.1. Inclusion criteria

The inclusion criteria consisted of:

- age over 18 years
- established diagnosis of histologically confirmed NAFLD
- without any other coexisting liver disease
- the patient's desire to participate in the study
- the ability to tolerate the study protocol

### 3.2. Exclusion criteria

The exclusion criteria consisted in:

- • The presence of other liver pathologies, namely:
  - viral hepatitis
  - alcohol-induced liver disease (we wanted an estimated consumption of less than < 20 mg/day for women and < 30 mg/day for men)
  -Hemochromatosis
  -Wilson’s disease
  - Hepatocellular carcinoma
  - Drug-induced liver disease
  - Patients with drugs that can interfere with the metabolism of octanoate or that can cause BFGNA (eg amiodarone, corticosteroids, methotrexate, tetracycline, valproic acid, zidovudine)
- The presence of other comorbidities (severe COPD, severe asthma, NYHA class III congestive heart failure and malabsorption syndromes)
- Presence of the pregnancy

- Hypersensitivity to 13C sodium octanoate
- Recent acute illness that required medical or surgical treatment (last 3 months)
- Patients who participated in other clinical trials.

## 4. The patient assessment protocol

Between October 2015 and September 2018, all patients evaluated in the Gastroenterology Department of the Bucharest University Emergency Hospital were included in the study according to the criteria mentioned above. Each patient underwent a testing protocol, which included a clinical examination, paraclinical explorations, standard abdominal ultrasound, and the 13C octanoate breath test. The definitive diagnosis of non-alcoholic steatohepatitis was established by liver biopsy with the characteristic histological changes. All tests were performed within 72 hours of study enrollment, with the notable exception of the liver biopsy, which was performed within the last 6 months.

## 5. Anamnesis and clinical exam

To begin with, all patients were subjected to a complete evaluation, including demographic data, which included age, sex, but also medical history, including measurement of height, weight, waist circumference. All subjects were administered the CAGE questionnaire to investigate the consumption of alcoholic beverages. The objective clinical examination was performed on devices and systems.

## 6. Paraclinical examination

The collection of biological samples was carried out under conditions of digestive rest and included: complete blood count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, γ-glutamyl transpeptidase (GGT), total bilirubin, direct bilirubin, alkaline phosphatase, international normalized ratio (INR). ), glucose, cholesterol, including high-density lipoprotein (HDL) and low-density lipoprotein (LDL) fractions, triglycerides, uric acid, serum iron and ferritin, urea, creatinine, HBs antigen, anti-HCV antibodies, anti- -mitochondrial, serum ceruloplasmin, alpha-feto-protein, antinuclear antibody, alpha1-antitrypsin, HbA1c. Devices used for sample analysis included CELL-DYN

(Abbot Diagnostics), ARCHITECT c8000 (Abbot Diagnostics), ACL TOP 500 (Laborator de instrumente), Access 2 Imunoassay System (Imunoassay System), analizor Dimension RXL (Dade Behring).

### 7. Assessment of hepatic steatosis and fibrosis

All patients were investigated by ultrasonography (US) using the Acuson S2000 machine (Siemens AG). The classification of "bright liver" or hepatic steatosis (NAFL) was based on a four-point hyperechogenicity scale depending on the difference between the densities of the liver. and the right kidney.

All included patients underwent histological evaluation by percutaneous liver biopsy within 6 months prior to inclusion in the studies.

The 13C-Octanoate breath test is a non-invasive test proposed as an alternative to liver biopsy as well as other non-invasive tests. Breath tests involve administering a substrate labeled with the stable carbon-13 isotope to the patient, which will then be metabolized, depending on the normal metabolism of each substrate, excreted and then measured. 13C octanoate has physical and chemical properties that may make it a suitable substrate for assessing hepatic mitochondrial β-oxidation by means of a breath assay. Octanoate [CH3(CH2)6CO2H], a medium-chain fatty acid, is rapidly absorbed from the intestine without prior incorporation into micelles and rapidly transported to the liver via the portal venous system. In hepatocytes, it undergoes β-oxidation in the mitochondria, producing acetyl coenzyme A (CoA). Acetyl CoA enters the Krebs cycle and is oxidized to CO2, which will then be transported through the systemic circulation to the lungs and eliminated in respiration [303]. Exhaled 13CO2 is captured in specially designed breath test bags, which are then analyzed using a 13C/12C infrared spectrometer.

The respiratory test was performed after a digestive rest of at least 12 hours, and during the test physical activity and any food consumption were prohibited. After collecting the first control air sample in a bag, each subject ingested 100 mg of stable, non-radioactive isotope-labeled 13C octanoate (sodium 13C-octanoate, Hanseaten-Apotheke) dissolved in 200 ml water. Breath samples were collected at baseline and 0, 15, 30, 45, 60, and 120 min after substrate administration. The analysis was performed within a maximum of 60 minutes after the collection of the last sample. The measured parameters included 13CO2 released through respiration following the metabolism of octanoic acid and consisted of: metabolic rate (PDR [%/h]) and total cumulative capacity of

octanoic acid metabolism as a function of time (cPDR [%]). The analyzer performed all measurements for a single patient simultaneously and automatically calculated PDR and cPDR for each time interval. The results were expressed in graphic form - curves of the 2 parameters according to the values obtained in different time intervals.

## 8. Statistical analysis

Data collection was done by entering them into the Microsoft Office Excel program, and after that, the MS Excel database was recoded and transposed into SPSS. The statistical analysis of the collected data was carried out using the Statistical Package for the Social Sciences (SPSS) program, version 23.0.

## 9. Results and discussions

81 patients were included in our study, of which 10 patients have liver fibrosis degree I, 8 patients degree II, 12 patients degree III and 19 patients degree IV and 32 patients do not have liver fibrosis. The distribution of patients according to the degree of liver fibrosis is not equal ($p<0.001$).

Among the 81 patients included in the study, 32 patients do not have liver fibrosis. 10 patients have liver fibrosis grade I, 8 grade II, 12 patients grade III and 19 patients grade IV. The distribution of patients according to the degree of liver fibrosis is not equal ($p<0.001$).

The degree of liver fibrosis correlates statistically significantly positively with the inflammatory activity ($rho=0.513$; $p<0.001$), i.e. the higher the degree of liver fibrosis, the higher the degree of inflammatory activity, and vice versa. (table no. 1) The age of the patients included in the study correlates statistically significantly positively with the degree of liver fibrosis ($rho=0.277$; $p=0.012$), i.e. the older the patients, the higher the degree of liver fibrosis, and vice versa. (table no. 1)

**Table no. 1. The correlations of age with the degree of hepatic steatosis, inflammatory activity and the degree of liver fibrosis**

| 1. | | Vârsta (ani) | Grad steatoză hepatică | Activitate inflamatore | Grad fibroză hepatică |
|---|---|---|---|---|---|
| **Vârsta (ani)** | Coef.corelație Pearson | 1 | 0,186 | 0,073 | 0,277* |
| | p value | | 0,096 | 0,517 | 0,012 |
| **Grad steatoză hepatică** | Coef.corelație Pearson | 0,186 | 1 | -0,053 | -0,185 |
| | p value | 0,096 | | 0,638 | 0,099 |
| **Activitate inflamatorie** | Coef.corelație Pearson | 0,073 | -0,053 | 1 | 0,513** |
| | p value | 0,517 | 0,638 | | <0,001 |
| **Grad fibroză hepatică** | Coef.corelație Pearson | 0,277* | -0,185 | 0,513** | 1 |
| | p value | 0,012 | 0,099 | <0,001 | |
| *. Corelația este semnificativă la nivelul 0,05 | | | | | |
| **. Corelația este semnificativă la nivelul 0,01 | | | | | |

The mean age of the patients included in the study differs statistically significantly according to the degree of liver fibrosis (p=0.016). The average age of patients without liver fibrosis is 44.63±13.45 years. Patients with degree I have an average age of 57.10±12.52 years, those with degree II have an average age of 50.25±9.04 years, those with degree III 51.83±7.31 years, those with degree IV they are 54.21±11.46 years old.

The distribution of patients according to the degree of liver fibrosis is statistically significantly different according to gender (p=0.033). Among the male patients, 11 have no liver fibrosis, 3 have liver fibrosis grade I, 2 have grade II, 9 patients have grade III, and 12 patients have grade IV. Of the women participating in the study, 21 do not have liver fibrosis, 7 have liver fibrosis grade I, 6 have grade II, 3 patients have grade III and 7 patients have grade IV.

Differences between men and women are influenced by socio-cultural factors (gender differences) which should be clearly delineated from biological sex differences. Both gender and sex differences underlie biological variation and disease variability, as well as disease progression.

Therefore, including gender and age in risk assessment, disease prevention, and treatment determination is crucial. [42].

In the current study, 81 patients were included, with no statistically significant differences in the proportion of each gender (p=0.505). Likewise, the distribution of patients according to gender and study group (hepatic steatosis/steatohepatitis) does not show statistically significant differences (p=0.083).

Singh et al. stated that abdominal circumference is an independent predictor of the degree of liver necrosis and inflammation. [43]. Stranges et al. demonstrated that abdominal circumference correlates better with GGT than body mass index. This correlation was demonstrated in both sexes. [44]. In the patients included in our study, a statistically significant positive correlation was identified between abdominal circumference and the degree of hepatic steatosis, the greater the abdominal circumference, the greater the degree of steatosis and vice versa (p=0.001). The AUC for abdominal circumference has a value of 0.664, which is acceptable in terms of the accuracy of using abdominal circumference as a predictor for steatosis.

The minimum cut-off value for abdominal circumference is 81.50 cm.

Our results reveal similar findings to those presented by Francazani et al, showing that abdominal circumference is associated with NASH, but does not appear as a statistically significant predictor in logistic regression analysis. Francazani believes that once hepatic steatosis appears, visceral obesity is no longer a major determinant in terms of the severity of liver damage. [45].

The body mass index was studied in order to establish a threshold value as an indicator for NASH [46]. In the 81 patients included in the study, we observed that the body mass index is statistically significantly higher in patients with steatohepatitis compared to those with hepatic steatosis (p=0.017). Also, body mass index correlates statistically significantly positively with inflammatory activity (p=0.004).

Insulin resistance is the main determinant of steatogenesis and probably liver disease. [47]In order to recognize this determinant, insulin resistance should be assessed in all patients included in the study. So, in all 81 patients included in the research, two parameters were evaluated: fasting blood glucose and the HOMA index, one of the validated methods for evaluating insulin resistance [48].

Our study demonstrates the positive correlation between blood glucose and the degree of liver fibrosis ($p=0.005$), as well as between the HOMA index and liver fibrosis ($p<0.001$), both blood glucose and the HOMA index registering higher values with the degree of fibrosis hepatic is higher.

Fasting blood glucose is not statistically significantly different in patients with steatohepatitis compared to those with hepatic steatosis ($p=0.089$), but the HOMA index is statistically significantly higher among patients with steatohepatitis ($p=0.022$).

Among the 81 patients included in the study, statistically significantly higher triglyceride values are recorded in those with steatohepatitis ($p=0.049$), and the triglyceride value increases significantly with inflammatory activity ($p=0.032$) and with the degree of liver fibrosis ($p=0.012$).

Our research demonstrates the relationship between GGT levels and liver damage, with a significant positive correlation between GGT levels and the degree of liver fibrosis ($p<0.001$) and inflammatory activity ($p<0.001$). Also, the average GGT in the patients with hepatic steatosis included in the study is statistically significantly ($p<0.001$) lower than the average GGT among the patients with steatohepatitis participating in the research.

In our study, MDA and GSH positively correlate statistically significantly with inflammatory activity ($p<0.001$) and the degree of liver fibrosis ($p<0.001$). MDA in patients with hepatic steatosis included in the study is statistically significantly ($p<0.001$) lower than in patients with steatohepatitis, and serum glutathione GSH is statistically significantly ($p<0.001$) lower in patients with steatohepatitis. GSH is the protection parameter of liver cells against oxidative stress. The increase in the values of free radicals, objectified in our study by the increased values of MDA, predominantly in patients with NASH, justifies the significantly low values of GSH in patients with steatohepatitis.

Inflammatory status was demonstrated by significantly higher values of inflammation parameters, reflected by PCR assessment. CRP correlates statistically significantly with inflammatory activity ($p<0.001$) and with the degree of liver fibrosis ($p<0.001$).

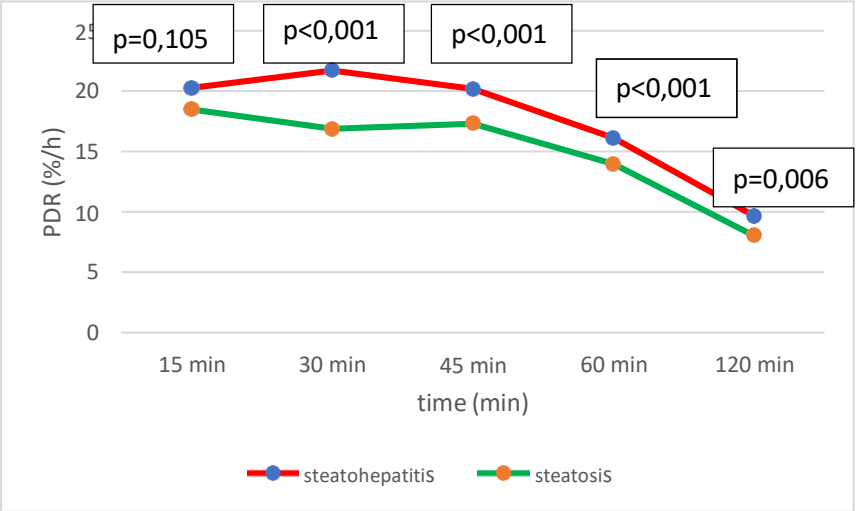
Also, in our study the mean PCR in patients with hepatic steatosis is statistically significantly (p<0.001) lower than the mean PCR among patients with steatohepatitis.

In this context, we evaluated the role of C13-labeled octanoic acid breath test (13C-OBT) as a surrogate marker of mitochondrial dysfunction in non-alcoholic steatohepatitis.

In our study, the results showed that 13C-OBT had good efficacy in identifying patients with nonalcoholic steatohepatitis from those with nonalcoholic fatty liver.

By examining the metabolic rate of octanoic acid at different time intervals, patients with non-alcoholic steatohepatitis had a significant increase in 13CO2 expiratory rate at (PDR) 30 min (p<0.001), 60 min (p<0.001) , 45 min (p<0.001) and 120 min (p=0.006 of on administration) compared to patients with simple hepatic steatosis. The results obtained are comparable to those of Fierbințeanu et al [303]. at time intervals of 30, 45, 60 and 120 min; in the current study, no statistically significant difference was revealed (p=0.105) in terms of PDR at 15 min.

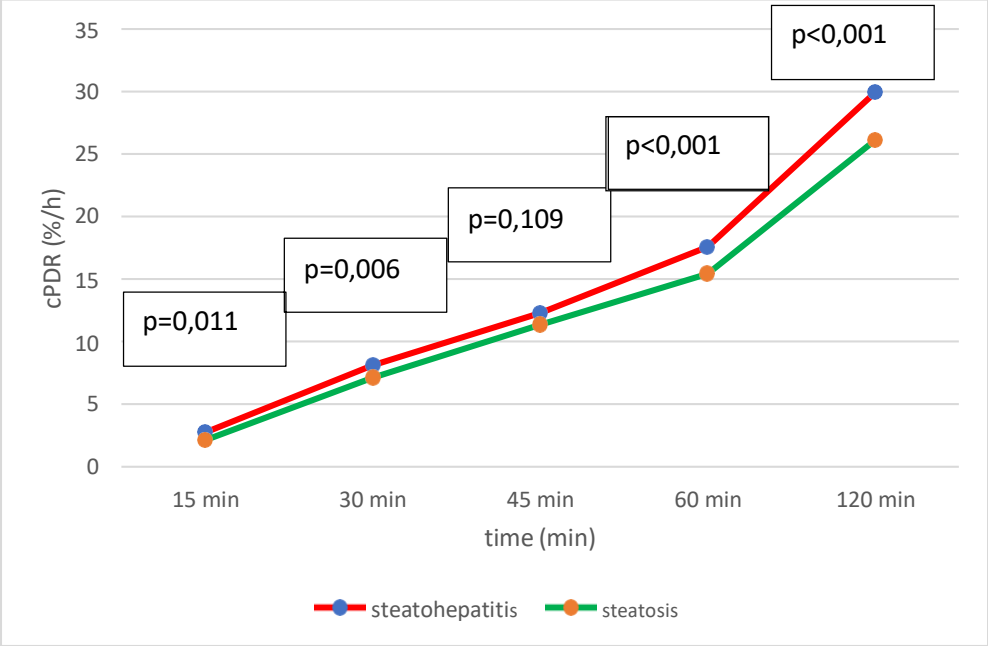
**Graph no. 1. PDR measurements by study group**




Regarding the total ability to metabolize octanoic acid, unlike patients with simple hepatic steatosis, patients with steatohepatitis had a higher ability to metabolize octanoic acid, expressed as percentage of cumulative 13CO2 recovery in breath (cPDR) at 15 min (p=0.011), 30 min (p=0.006), 60 min (p<0.001) and 120 min (p<0.001). Differences between patients with simple hepatic steatosis and those with non-alcoholic steatohepatitis have

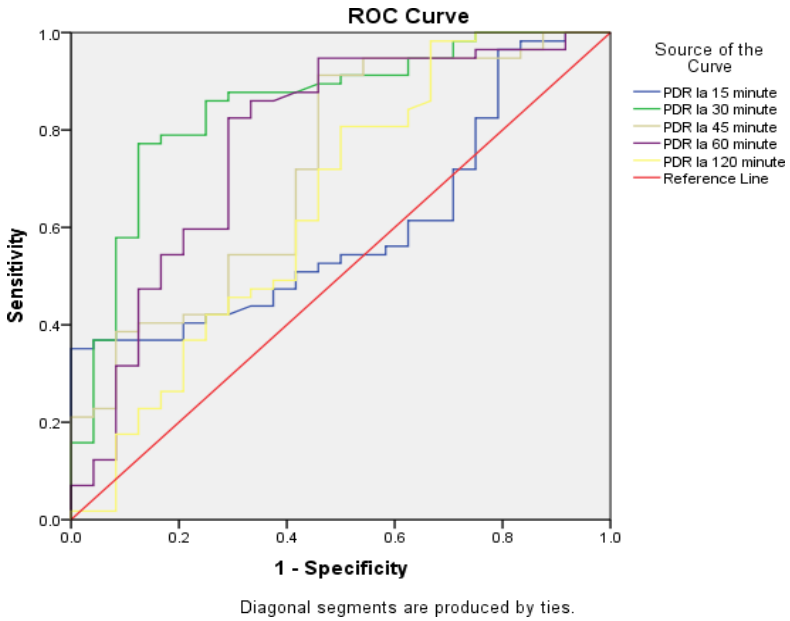
increased with time points, with the largest difference observed for cPDR at 120 min (26.12±3.77% vs. 29.92±3.72%). The characteristic curves regarding the speed and total capacity of metabolizing octanoic acid allowed the differentiation of patients with non-alcoholic steatohepatitis from patients with simple hepatic steatosis.

**Graph no. 2. cPDR measurements by study group**



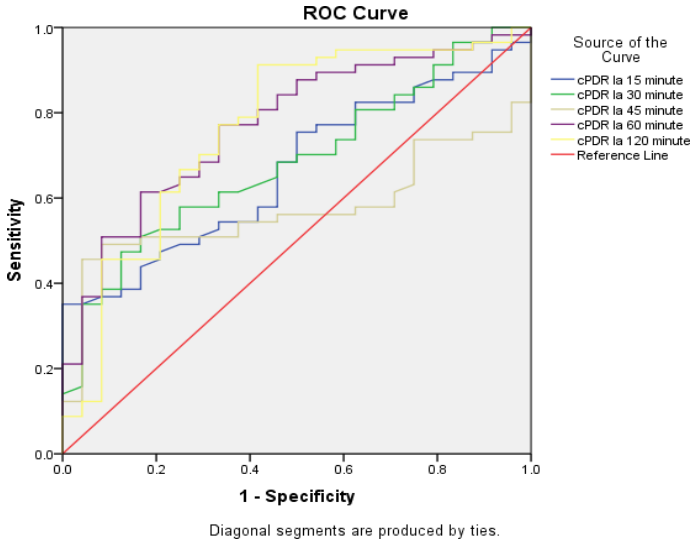

In order to evaluate the performance of OBT in differentiating patients with simple hepatic steatosis and those with non-alcoholic steatohepatitis, we studied the ROC curve. The best diagnostic power of nonalcoholic steatohepatitis was proven by PDR at 30 min. with a threshold value of 17.26%, the AUC for this parameter recorded a good value of 0.848, with a sensitivity of 87.7% and a specificity of 70.8, the positive predictive value being 87.7% and the negative of 70.8%.

**Graph no. 3. ROC curve for PDR 15, 30, 45, 60 and 120 min in Steatohepatitis**




Total metabolic capacity or cumulative dose (cPDR) was acceptable at 60 min and 120 min (AUC=0.772, respectively 0.765), at threshold values of 15.72% and 24.45%, respectively. Sensitivity values were good, 80.7% at 60 min and 94.7% at 120 min, but specificity values were low (58.3% at 60 min and 41.7% at 120 min).

**Graph no. 4. ROC curve for cPDR 15, 30, 45, 60 and 120 min in steatohepatitis**



The present study has certain limitations. The small number of patients led, in some cases, to obtaining results with statistical significance, but without clinical significance, which does not exclude the possibility that the clinical relevance can be supported by the results obtained by including a larger number of patients in the study .

## 10. Conclusions

1. The average age of patients with non-alcoholic steatohepatitis is statistically significantly ($p=0.002$) higher compared to those with simple hepatic steatosis.
2. Gender and age differences underlie the variability of the disease and its progression, so patient demographic characteristics should be included in the risk assessment and prognosis determination of NAFLD.
3. The degree of liver fibrosis correlates statistically significantly positively with the age of patients ($p=0.012$) and inflammatory activity ($p<0.001$), the stage of liver disease being more severe at older ages.
4. The degree of liver fibrosis differs significantly ($p=0.033$) depending on the gender of the patients, men having an advanced stage of liver fibrosis.
5. In order to determine the prognosis of liver disease, the staging of steatosis, inflammatory activity and liver fibrosis, together with the demographic characteristics of the patients, form the basis of the prediction model of the evolution of nonalcoholic fatty liver.
6. The cumulative prevalence of obesity and overweight -79% (52%, 27%, respectively) among the patients included in the research is higher than that recorded in the general population.
7. Body mass index and abdominal circumference are significantly higher in patients with non-alcoholic steatohepatitis compared to those with simple steatosis ($p=0.017$, respectively $p=0.015$), and body mass index is positively correlated ($p=0.004$) cu

with inflammatory activity and abdominal circumference with the degree of hepatic steatosis ($p=0.001$).

8. Obesity, especially visceral obesity, represents one of the most important risk factors for non-alcoholic steatohepatitis, abdominal circumference thus remains the simplest and most used way of assessment, being an independent predictor of the degree of liver necrosis and inflammation.
9. The longitudinal diameter of the spleen of the patients included in the study correlated positively with the degree of inflammatory activity ($p<0.001$) and with the degree of liver fibrosis ($p<0.001$), being significantly higher ($p<0.001$) among patients with non-alcoholic steatohepatitis compared to those with simple steatosis.
10. Fasting blood glucose and the HOMA index were significantly positively correlated with the degree of liver fibrosis ($p=0.005$, respectively $p<0.001$), the HOMA index being statistically significantly higher among patients with non-alcoholic steatohepatitis ($p=0.022$).
11. Serum triglyceride values were significantly higher in patients with non-alcoholic steatohepatitis ($p=0.049$), increasing significantly with the inflammatory activity ($p=0.032$) and with the degree of liver fibrosis ($p=0.012$).
12. Our study confirms the multifactorial etiology of nonalcoholic fatty liver, involving genetic and metabolic factors: obesity, insulin resistance, dyslipidemia, type 2 diabetes and cardiovascular diseases.
13. The analysis of the ROC curves in order to evaluate the ability of the glycemic and lipid profile to detect the presence of steatohepatitis revealed a poor performance, the use of biological tests for the evaluation of glycemic and lipid profiles being insufficient as non-invasive tests for the prediction of non-alcoholic steatohepatitis.

14. GGT, as a marker of oxidative stress, along with MDA and GSH was positively correlated with the degree of liver fibrosis and inflammatory activity ($p<0.001$). CRP correlated with inflammatory activity ($p<0.001$) and with the degree of liver fibrosis ($p<0.001$), being significantly higher in patients with non-alcoholic steatohepatitis ($p<0.001$).
15. Using the ROC curve to study the ability of PCR to detect the presence of steatohepatitis, we recorded an AUC value for PCR of 0.840, considered very good in terms of the accuracy of using PCR as a predictor for steatohepatitis, with a sensitivity value of 66.7% and specificity of 91.7%.
16. PCR represents an independent predictor of hepatic steatosis, since the diagnosis of NAFLD phenotypes can be included in the algorithm, PCR can differentiate steatohepatitis from simple, non-progressive steatosis. Prediction of NAFLD progression can be analyzed based on markers of inflammation, confirming previous hypotheses regarding the role of oxidative stress and inflammation alongside insulin resistance. in the progression of the disease.

17. Respiratory tests based on a C13-labeled substrate, with the well-defined metabolism of the substrate at the level of hepatocytes, allow the establishment of liver dysfunction in the context of NAFLD.

18. By examining the rate of metabolism of octanoic acid, patients with non-alcoholic steatohepatitis had a significant increase in the concentration of $13CO_2$ in the exhaled air, at the time intervals (PDR) 30 min ($p<0.001$), 45 min ($p<0.001$ ), 60 min ($p<0.001$) and 120 min ($p=0.006$) compared to patients with simple hepatic steatosis.

19. Regarding total octanoic acid metabolizing capacity, patients with steatohepatitis demonstrated a higher octanoic acid metabolizing capacity, expressed as cumulative percentage of $13CO_2$ recovery in expired air (cPDR) at 15 min ($p=0.011$ ), 30 min ($p=0.006$), 60 min ($p<0.001$) and 120 min ($p<0.001$). The differences between patients with simple hepatic steatosis and those with non-alcoholic steatohepatitis increased

with increasing time intervals since substrate administration, the greatest difference being observed for cPDR at 120 min (26.12±3.77% vs. 29 .92±3.72%).

20. Using the ROC curve, it was revealed that the best performance for the diagnosis of nonalcoholic steatohepatitis was present for PDR at 30 min; a threshold value of 17.26%, the AUC was 0.848, with a sensitivity of 87.7% and a specificity of 70.8, with a positive predictive value of 87.7% and a negative predictive value of 70.8%.
21. Regarding the total ability to metabolize octanoic acid (cPDR), the best parameters were cPDR at 60 min and at 120 min (AUC=0.772, respectively 0.765), at threshold values of 15.72%, respectively 24.45 %; Sensitivity values were 80.7% at 60 min and 94.7% at 120 min, but with low specificity values (58.3% at 60 min and 41.7% at 120 min).
22. $^{13}$C-OBT has good efficacy in identifying patients with non-alcoholic steatohepatitis among patients with NAFLD. A good performance of the octanoic acid breath test is noted in predicting the increased degree of hepatic steatosis, along with a good correlation of the PDR and cPDR parameters and the degrees of liver fibrosis.
23. The octanoic acid breath test is a valuable non-invasive diagnostic test that can be widely applied in NAFLD, to increase its potential there is a need for standardization of performance and interpretation.
24. The good tolerance, the absence of adverse reactions, the functional nature of the mitochondrial exploration determines the continuation of the investigations regarding its incorporation into the non-invasive diagnosis algorithm of non-alcoholic steatohepatitis, a desire still unfulfilled in hepatology.

**Personal contributions**

The main objective of this PhD thesis was to evaluate the effectiveness of the 13C-Octanoate breath test (OBT), as a surrogate marker of mitochondrial function, in differentiating patients with NASH from patients with simple steatosis (NAFL). Thus, I started researching the scientific literature and the papers appeared: "The role of antioxidant therapy in non-alcoholic steatohepatitis", "The Role of Noninvasive 13C- Octanoate Breath Test in Assessing the Diagnosis of Nonalcoholic Steatohepatitis", where, together with my collaborators, I summarized the main pathophysiological relationships of this condition, as well as current notions about management. Next, my personal contribution materialized by demonstrating the "non-invasive algorithm for risk stratification in patients with non-alcoholic fatty liver disease" by creating a non-invasive evaluation method, a 13C Octanoate breath test, which could in the future replace performing liver biopsy in these patients. Last but not least, my own contribution also consisted in going through the stages that led to the fulfillment of the final goal of this doctoral research paper, starting with the creation of the study methodology and the patient evaluation protocol to discover new non-invasive strategies that allows assessment of the full spectrum of NAFLD.

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**ANEXE**

## LISTA CU LUCRARILE STIINTIFICE PUBLICATE

### Articole publicate in reviste de specialitate

- Din tematica tezei de doctorat

⇒ Ana Calin-Necula, V.Enciu, P. Ologeanu, A.C.Moldoveanu, Carmen Fierbinteanu-Braticevici. The correlation between Body Mass Index and histological features of Nonalcoholic Fatty Liver Disease, ROM. J. INTERN. MED., 2023, 0, 0,1-15, DOI: 10.2478/rjim-2023-0011 BDI; (Capitol IV-p.82,p106-113)
http:// DOI: 10.2478/rjim-2023-0011

⇒ Carmen Fierbinteanu-Braticevici, Ana-Maria Calin-Necula, V.T. Enciu, L. Goran, Anca Pantea Stoian , Ioan Ancuta, O. Viasu , A.C. Moldoveanu. The Role of Noninvasive 13C- Octanoate Breath Test in Assessing the Diagnosis of Nonalcoholic Steatohepatitis *Diagnostics* 2022, *12*, 2935. ISI-IF: 3,992 (Capitol III– p. 85-p.88)
https://doi.org/10.3390/ diagnostics12122935

⇒ Ana Calin-Necula, A. Moldoveanu, R.Peagu, O. Viasu, R. Sararu, A. Petrisor, G. Oprea ,E. Sarbu, L.Tribus, R. Usvat, C.Fierbinteanu-Braticevici. Rolul terapiei antioxidante in steatohepatita non-alcoolica. Internal Medicine, vol.XIV no.5, 2017, pp.15-23 BDI ( Capitol VIII -p.59-p.73 ).
http://www.medicina- interna.ro/articol.php?articol=1002&lang =ro

⇒ Carmen Fierbinteanu-Braticevici, V. T. Enciu, **Ana-Maria Calin-Necula**, I. R. Papacocea, A.C. Moldoveanu, A Comparison of $^{13}$C-Methacetin and $^{13}$C-Octanoate Breath Test for the Evaluation of Nonalcoholic Steatohepatitis, J Clin Med. 2023 Mar 10;12(6):2158. doi: 10.3390/jcm12062158. **ISI- IF: 4.964**
http:// doi: 10.3390/jcm12062158

- **Articole cu alta tematica**

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