UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

PhD THESIS

CURRENT METHODS USED FOR ASSESSING LIVER STEATOSIS

- ABSTRACT-

SCIENTIFIC COORDINATOR:

PROFESSOR DAN IONUȚ GHEONEA, MD, PhD

PhD STUDENT:

BECHERU (LĂPĂDAT) ALINA-MARIA

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents an ectopic accumulation of fat in the liver parenchyma that is not due to excessive ethanol consumption. The incidence of NAFLD has increased rapidly in recent decades, becoming one of the most common causes of chronic liver damage. This is mainly due to the overall increase in the incidence and prevalence of obesity due to increased consumption of carbohydrates and lipids, as well as due to a predominantly sedentary lifestyle.

The term NAFLD encompasses a wide range of evolving liver pathologies and various therapeutic possibilities and is usually associated with dyslipidemia, hypertension, obesity, insulin resistance / type 2 diabetes - a group of chronic conditions that make up the metabolic syndrome and indicate an increased cardiovascular risk.

2. Current state of knowledge

At present, the exact value of the overall prevalence of NAFLD remains unknown, as there is a marked variability (3% - 45%) between the estimates provided by various studies mainly due to differences in ethnicity, diet and sensitivity of the diagnostic methods (serum liver enzymes, imaging investigations, liver biopsy). Currently, the gold standard for diagnosing and assessing NAFLD is liver biopsy. However, the main disadvantage of this technique is its invasive nature. Current data in the literature indicate the possibility of using magnetic resonance spectroscopy (MRS) as a non-invasive alternative, able to quantify the level of triglycerides accumulated in the liver parenchyma.

NAFLD most commonly develops in subjects of Hispanic descent. In the Asian population, the prevalence of NAFLD is constantly increasing and, not infrequently, the disease can be detected among people with normal body mass index (BMI) values.

The diet, especially the high-fat one, has been and continues to be considered an independent risk factor in the development of NAFLD. Diets frequently adopted in Western countries, rich in red meat, pastries, refined cereals and sugary drinks are often associated with an increased risk of developing metabolic syndrome and, implicitly, NAFLD.

Initially, it was thought that simple hepatic steatosis, also called non-alcoholic fatty liver (NAFL) has a benign evolution that associates minimal risks of sequelae. According to this theory, non-alcoholic steatohepatitis (NASH) is the only clinically relevant form of the disease. Current information in the literature questions this aspect and indicates the presence of an initial dynamic phase in which the liver parenchyma oscillates between the two diseases: NAFL and NASH.

Subjects with NALFD are usually asymptomatic. Not infrequently, the disease is detected through an increased level of liver enzymes or with the help of imaging diagnostic tools performed during a routine check-up. NAFLD is mainly considered in patients with persistently elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) accompanied by the presence of hepatic steatosis detected by imaging techniques such as ultrasonography (US) or computed tomography (CT), in the absence of drug and alcohol consumption, as well as in the absence of viral hepatitis markers. An important aspect to note is that patients with NASH who have developed liver cirrhosis have low levels of ALT and AST. Thus, the existence of NASH cannot be ruled out under conditions of normal ALT values in patients with advanced liver fibrosis.

Patients with NAFLD usually have an AST / ALT ratio of less than 1. However, as the disease progresses and the fibrotic lesions become more pronounced, liver cirrhosis occurs, with a decrease in ALT and, respectively, an increase in AST values so that the AST / ALT ratio is reversed and has values greater than 1. It is important to note that AST values may exceed ALT values at least twice in case of hepatic steatosis caused by excessive alcohol consumption.

Staging of hepatic steatosis is performed according to the percentage of affected hepatocyte cells:

- grade 0: less than 5% of hepatocytes are affected;
- grade I: between 5-33% of hepatocytes are affected;
- grade II: between 34-66% of hepatocytes are affected;
- grade III: over 66% of hepatocytes are affected.

Liver biopsy allows the examination of the liver parenchyma in order to evaluate the inflammatory activity of the disease, but also the progression of fibrotic lesions to liver cirrhosis. In patients with NAFLD, the liver damage is diffuse, uneven (to varying degrees). Taking this into consideration, the average percentage of hepatic steatosis is obtained by calculating the arithmetic mean of all individual percentages obtained through liver biopsy from different locations.

Most of the therapeutic measures applicable to patients with NAFLD aim to change their lifestyle and diet.

3. Personal contribution

At the beginning of the study, I set myself the main objective to evaluate the effectiveness of MRS in diagnosing and staging of NAFLD by relating the results obtained through this imaging method to those provided by liver biopsy. Numerous serum parameters will also be studied among these patients. Thus, highlighting statistically significant associations between the NAFLD grade and

the evaluated parameters is an important component of the present study, as I believe that possible connections between these factors can significantly influence the way that NAFLD is diagnosed and can help identify the population at risk among these patients.

In the second part of the study, I chose to evaluate the effectiveness of a well-established set of therapeutic measures by examining a different group of patients using MRS both at the time of inclusion in the study and after completing the recommendations provided in the therapeutic program. Thus, I believe that the study presented in detail in this paper will have important implications in the therapeutic management of patients with NAFLD.

For a proper development of the research activity, I obtained the approval of the Ethics Commission of the University of Medicine and Pharmacy of Craiova for the current study. All subjects included and presented in this paper expressed their consent to participate in the study.

The study was conducted prospectively during 2017-2020 and included a total number of 73 patients divided into two groups as follows:

- group I: 38 patients;
- group II: 35 patients.

The first study group (consisting of 38 patients) is represented by subjects in which I evaluated the usefulness of MRS (a non-invasive method) in diagnosing and staging of NAFLD. The persons included in this study group were hospitalized within the Gastroenterology Clinic and, respectively, within the Surgery Clinic II of the Emergency Clinical County Hospital Craiova. The high-performance MRS imaging investigations took place within the Medical Imaging Department of the University of Medicine and Pharmacy of Craiova. Also, biopsy samples from the liver parenchyma were collected at the Surgery Clinic II of the Emergency Clinical County Hospital Craiova. The histopathological samples were subsequently evaluated at the Center for Studies of Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of

Craiova. Due to the fact that hepatic steatosis affects the structure of the liver in a diffuse and heterogeneous manner, in varying degrees, it is important to note that the liver biopsy samples were collected from the right hepatic lobe from a variable number of locations (minimum three).

Throughout the study, the Center for Studies of Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova benefited from a high-performance microscope (NIKON 90i). The slides obtained from liver biopsy samples were stained with hematoxylin-eosin and subsequently digitally examined using the Image-Pro Plus version 7 application developed by Media Cybernetics, USA. Thus, after running this software, I individually uploaded each hematoxylin-eosin stained slide. Subsequently, the areas of fat in the uploaded images were marked and delimited by the rest of the tissue. The percentage of fat was calculated by relating the area occupied by the fatty tissue in each image to the entire surface of the image. The final fat percentage was obtained by calculating the arithmetic mean of all fat percentage values obtained in that patient. Among the patients included in the first study group, we evaluated the following parameters:

- age;
- sex;
- fat fraction (FF) indicated by liver biopsy;
- the degree of NAFLD indicated by liver biopsy;
- FF indicated by MRS;
- the degree of NAFLD indicated by MRS;
- serum triglyceride values;
- serum values of total cholesterol;
- serum values of transaminases (ALT, AST);
- fasting blood glucose values;
- the presence / absence of diabetes;

– BMI values;

– BMI degree.

The second study group (consisting of 35 patients) is represented by subjects with NAFLD, in which the effectiveness of therapeutic measures was evaluated through MRS. The patients included in this study group were hospitalized in the Gastroenterology Clinic of the Emergency Clinical County Hospital Craiova. The high-performance MRS imaging investigations were performed within the Medical Imaging Department of the University of Medicine and Pharmacy of Craiova both at the time of inclusion in the second study group and after completing the recommendations provided in the therapeutic program. The treatment applied to the patients in the second study group was performed for 6 months and included a hepatoprotective dietary supplement in association with changes in diet and lifestyle. Among the patients included in the second study group, we evaluated the following parameteres:

– age;

– sex;

– FF indicated by MRS at the time of inclusion in the study;

– the degree of NAFLD indicated by MRS at the time of inclusion in the study;

– FF indicated by MRS at the end of treatment;

– the degree of NAFLD indicated by MRS at the end of the treatment;

– did the degree of NAFLD decrease ?;

– how much did the FF decrease ?.

The characteristics of all patients included in the first study group are presented according to the degree of NAFLD in *Table 1*.

Investigated parameter (mean value±SD)		Grade 0	Grade I	Grade II
Age (years)	**T**	59±6,20	45,70±8,57	56,06±9,85
	M	57,33±3,21	46,58±9,60	52,90±10,08
	W	61,50±10,60	43,60±5,68	61,33±7,42
FF indicated by liver biopsy (%)	**T**	3±1	22,88±7,12	48,93±10,25
	M	3±1	22,25±6,86	45,50±10,27
	W	3±1,41	24,4±8,32	54,66±7,89
FF indicated by MRS (%)	**T**	3,6±0,54	23±6,95	49,93±9,58
	M	3,33±0,57	22,41±6,84	46,80±9,87
	W	4±0	24,4±7,82	55,16±6,88
Serum triglycerides (mg/dL)	**T**	155,2±3,96	210,47±43,09	296,37±29,72
	M	152,66±2,51	210,41±48,23	286,50±27,80
	W	159±1,41	210,6±32,08	312,83±27,17
Serum total cholesterol (mg/dL)	**T**	265,80±29,54	255,35±75,57	252,37±60,23
	M	257,33±38,42	242,91±65,76	266,50±61,47
	W	278,5±0,7	285,2±96,81	228,83±54,95
Serum ALT (U/L)	**T**	31,60±2,60	49,05±15,38	50,12±18,51
	M	32,66±2,30	46,91±15,35	50,90±19,73
	W	30±2,82	54,2±15,84	48,83±17,98
Serum AST (U/L)	**T**	29±2,34	41,47±11,97	42,81±15,62
	M	30,33±2,08	39,25±11,74	43,90±15,84
	W	27±0	46,80±12	41±16,56
Fasting blood glucose (mg/dL)	**T**	99,6±3,04	111,41±36,23	131,62±35,15
	M	98,66±3,78	116,25±39,43	131,30±35,16
	W	101±1,41	99,80±27,10	132,16±38,49

BMI (kg/m^2)	**T**	25,2±2,38	32±4,96	35,50±4,83
	M	26,33±2,51	31,58±4,83	35±5,53
	W	23,50±0,70	33±5,70	36,33±3,66

Table 1. The mean values ± standard deviation (SD) of all the investigated parameters among the patients included in the first study group according to the degree of NAFLD. T - Total, M - Men, W - Women

Among the patients included in the first study group, I assessed possible statistically significant associations between the following parameters:

– between the degree of NAFLD indicated by liver biopsy and the degree of NAFLD indicated by MRS ($p < 0.001$);

– between the degree of NAFLD (liver biopsy / MRS) and the serum triglyceride level ($p < 0.001$);

– between the degree of NAFLD (liver biopsy / MRS) and BMI ($p < 0.001$);

– between the degree of NAFLD (liver biopsy / MRS) and the level of total serum cholesterol ($p = 0.461$);

– between the degree of NAFLD (liver biopsy / MRS) and the serum level of ALT ($p = 0.430$);

– between the degree of NAFLD (liver biopsy / MRS) and the serum level of AST ($p = 0.346$).

The distribution of patients included in the second study group according to the degree of NAFLD at the time of inclusion in the study and after completing the recommendations provided in the therapeutic program is presented in *Table 2*.

The therapeutic measures applied to the patients in the second study group resulted in a reduction of fat content in hepatocytes in 24 patients (68.57%), while in the remaining 11 patients there was an increase in the amount of intrahepatic adipose tissue.

Number of patients		Grade 0	Grade I	Grade II	Grade III
At the time of inclusion	**T**	0	9	23	3
	M	0	9	15	1
	W	0	0	8	2
After completing treatment	**T**	0	17	18	0
	M	0	11	14	0
	W	0	6	4	0

Table 2. Distribution of all patients included in second study group, regardless of sex, according to the degree of NAFLD both at the time of inclusion in the study and at the end of treatment. T - Total, M - Men, W - Women

In the second study group, the FF decrease was, on average, around 13.49% (± 7.24%), registering values between 4.15% and 27.96%. Among men, the FF decrease was, on average, around 15.96% (± 7.48%), registering values between 6.01% and 27.96%. Among women, the FF decrease was, on average, around 10.02% (± 5.52%), registering values between 4.15% and 18.55%.

In the first study group, the patients' reluctance to liver biopsy was the main limitation. This was mainly due to the invasive nature of the technique, but also due to the discomfort created during the procedure. The main consequence of this was the inclusion of a smaller number of patients, but sufficient in my opinion to illustrate the accuracy of MRS in diagnosing and staging NAFLD using a non-invasive alternative to liver biopsy.

In the second study group, the main factors that limited the inclusion of a larger number of patients were mainly the increased duration of treatment (six months) and low adherence to treatment.

4. Conclusions

– The present study highlighted a statistically significant association between the degree of NAFLD indicated by liver biopsy and the degree of NAFLD indicated by MRS ($p < 0.001$);

– The current study highlighted a statistically significant association between the degree of NAFLD (liver biopsy / MRS) and the serum triglyceride level ($p < 0.001$);

– The present study highlighted a statistically significant association between the degree of NAFLD (liver biopsy / MRS) and BMI ($p < 0.001$);

– Regarding the mean age, all patients with grade 0 NAFLD had the highest values. Also, among female subjects, mean age values were similar between those diagnosed with grade 0 NAFLD and those diagnosed with grade II NAFLD but significantly higher than the mean values recorded in patients with grade I BFGNA;

– The mean value of FF indicated by both liver biopsy and MRS is around 3% among subjects diagnosed with grade 0 NAFLD, around 22% among patients diagnosed with grade I NAFLD and around 46% among subjects diagnosed with grade II NAFLD;

– The mean serum values of triglycerides are directly proportional with the degree of NAFLD. However, this trend is not applicable for the average values of total serum cholesterol;

– Mean serum ALT and AST values increased significantly in the group of subjects diagnosed with grade I NAFLD compared to the group of patients diagnosed with grade 0 NAFLD. However, the mean serum values of ALT and AST were simillar between patients diagnosed with grade I and grade II NAFLD. This tendency is found only in case of male subjects, but not in case of female patients;

– The mean fasting blood glucose values appear to be directly proportional with the degree of NAFLD, most likely in association with the increased incidence of diabetes with increasing degree of NAFLD. This tendency is found only in case of male subjects, but not in case of female patients;

– The average BMI values appear to be directly proportional with the degree of NAFLD, both in case of male and female patients;

– There is a significantly higher effectiveness of therapeutic measures among women compared to men reflected, first of all, by the difference between the average values recorded at the time of inclusion in the study and the average values recorded at the end of the therapeutic measures. Thus, the mean FF value decreased by 3.26% in men and by 11.89% in women;

– Although the fat content inside the hepatocytes decreased in 24 patients (over two thirds), the decrease in NAFLD grade was recorded only in 16 patients (45.71%). The explanation for this phenomenon is that, although the FF decreased, it was not enough to cause a decrease in NAFLD grade.