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# LIVER FIBROSIS ASSESSMENT IN PATIENTS WITH TYPE II DIABETES MELLITUS - CLINICAL AND BIOLOGICAL CORRELATIONS

**PHD THESIS SUMMARY**

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# I. General Part

## 1. Introduction

This PhD thesis approaches a topic of interest, with important social and economic implications, i.e., non-alcoholic fatty liver (NAFL) in patients with type 2 diabetes mellitus (T2DM). Over the last decade, the sedentary lifestyle and the diet rich in saturated fats and high glycemic index carbohydrates led to dramatic changes in the general metabolic status of the population. The incidence of metabolic dysfunctions, such as obesity, NAFL and T2DM, has increased at an alarming pace globally, both in developed countries and in developing countries. NAFL currently has a global prevalence of 25.2%, with the highest rates in Southern America (31%) and the Middle East (32.5%), followed by Asia (27%), the United States of America (24%) and Europe (23%) [1]. Moreover, the high prevalence of NAFL is accompanied by the progressive rise in the obesity and T2DM epidemics, disorders that coexist in most patients with metabolic dysfunctions. NAFL and T2DM are two related pathologies, sharing identical risk factors, as well as a similar epidemiology and physiopathology.

The association of NAFL with the mortality and morbidly from metabolic, cardiovascular, renal and liver diseases raises serious challenges for clinicians throughout the world with regards to the prevention, diagnostic and treatment of this disorder. Hence, the establishment of a diagnostic conduct for the timely detection of advanced fibrosis, the major predictor for NAFL, becomes of essence, not only in the liver disease prognosis, but also with regards to the onset of systemic manifestations, such as cardiovascular events, stroke, metabolic complications etc. [2].

Liver fibrosis staging in T2DM patients gained in relevance when the first cases secondary to a respiratory infection caused by Severe acute respiratory syndrome coronavirus 2 (SARS-COV2) were described in Wuhan Region, in China. This viral infection shortly escalated into a pandemic [3, 4]. As of that point in time, the whole medical scientific community have concentrated their efforts towards the identification of the risk factors associated to the progression towards severe forms and decease of the SARS-COV2 infection. Large population studies throughout the world have described metabolic disorders, particularly obesity and T2DM, as major risk factors for the severe form of COVID-19 [4-6]. Moreover, it has been shown that the presence of MS-FL is associated with the severe forms of COVID-19, the patients

exhibiting a slower viral clearance and a higher probability of developing liver cytolysis during hospital stays [7].

The arguments above underpinned the two studies comprised in this doctoral thesis, described in detail in the chapters to follow.

# 2. Non-alcoholic fatty liver and type 2 diabetes mellitus

## 2.1 Definition and epidemiology

First introduced by Ludwig et al in 1980 [8], the term of "non-alcoholic fatty liver" currently represents the most common cause of chronic liver disease throughout the world, with a global prevalence of 25.2% [9]. It is traditionally defined as the presence of liver steatosis in more than 5% of the hepatocytes, in the absence of other liver impairment causes, i.e., ethanol consumption, viral or drug-induced hepatitis, hereditary liver diseases etc. [10].

Nonetheless, the term of NAFL fails to render the importance of the metabolic etiology and of insulin resistance, the primary determinants of liver steatosis development, and its definition does not include important risk factors contributing to its physiopathology. Hence, an international expert consensus-based definition was proposed for the term, i.e., that of metabolic syndrome-related fatty liver (MS-FL). The term comprises the whole pathological spectrum of the disorder and one of the following 3 criteria: overweight/obesity, the presence of T2DM or proof of dysfunction disorder

The growing prevalence of NAFL is accompanied by the progressive rise of the obesity and T2DM epidemics, disorders that coexist in most patients with metabolic dysfunctions. NAFL and T2DM are two related pathologies, sharing identical risk factors, as well as a similar epidemiology and physiopathology.

## 2.2 Physiopathology

### 2.2.1 Non-alcoholic fatty liver - a consequence of type 2 diabetes mellitus

Type 2 diabetes mellitus is one of the strongest predictors of NAFL progression towards the advanced forms of liver disease, such as NASH or cirrhosis [11]. In the absence of T2DM, approximately 15% of NAFL individuals will develop NASH, with a high risk of progression towards advanced forms. In the presence of T2DM, this risk is at least threefold higher, Koehler et al describing an advanced fibrosis rate of 18% in a population of diabetic patients, using transient elastography as reference method [12].

### 2.2.3 Type 2 diabetes mellitus - a consequence of non-alcoholic fatty liver

The prevalence of type 2 diabetes mellitus in SS and NASH is estimated at around 23% and 44%, rates that are much higher as compared to the prevalence of T2DM in the general population, which is of 8.5% [13].

The complexity of the interactions between NAFL, the quantity of visceral fat and the insulin resistance makes it difficult to accurately identify the mechanisms that trigger higher T2DM risks in NAFL patients. The trigger of the physiopathology cascade most probably is the higher amount of perivisceral adipose tissue, which initiates several processes, such as the release of free fatty acids and proinflammatory adipokines, leading to the development of insulin resistance, which plays a key role in the pathology of this association [14].

## 2.3 Diagnosis and follow-up

### 2.3.1 Invasive assessment methods

Liver biopsy is regarded as the gold standard in the diagnosis and histological assessment of NAFL. Nonetheless, because it is invasive, it is not suitable for screening purposes and it cannot be implemented at early stages in the evolution of the disease [15].

### 2.3.2 Non-invasive assessment methods

The non-invasive methods for the assessment of liver fibrosis are divided based on their approach: methods relying on a biological approach, i.e., the quantification of serum biomarkers, and methods relying on a physical approach, i.e., the measurement of liver stiffness [16].

*Methods relying on the quantification of serum biomarkers*

The NFS (NAFLD Fibrosis Score) has proven to be a good diagnostic tool as a liver fibrosis prognosis marker, especially for advanced fibrosis, which was excluded with a negative predictive value of 93% by applying a low cutoff score and a high positive predictive value, of 90%. The FIB-4 (Fibrosis-4) score, exclusively comprising routinely determined biomarkers, such as AST, ALT and thrombocytes, was initially developed for the assessment of liver fibrosis

in patients with chronic hepatitis C virus (HCV). Similarly, the APRI ((AST)-to-platelet ratio index)) score was initially developed as a predictor of significant fibrosis (F2) among chronic HCV patients [17].

*Methods relying on quantification of liver stiffness*

These methods are divided into two main types, i.e., strain elastography (SE) or real-time elastography, and shear wave elastography (SWE). SE is a qualitative method to assess liver tissue stiffness reliant on manual compression, whereas SWE is a method to quantitatively assess liver tissue stiffness. The techniques using SWE include transient elastography (TE), the only non-imagistic method reliant on the existence of an external mechanical impulse.

Unlike TE, Shear Wave (pSWE) ultrasound elastography (ARFI) and 2D Shear Wave elastography (2D SWE) are integrated in the ultrasound systems [18].

### 2.4 Treatment

Regardless of the presence or absence of T2DM, currently, there is no standardized pharmacological therapy for NAFL, approved by international bodies. The hygiene and diet therapy, with a focus on the change of the lifestyle is the key point in the management of this disorder. Physical activity offers very good results, including the regression of liver fibrosis and of NASH, an average of 150-200 min. of minutes of moderate-intensity physical activity/week, divided into 3-5 sessions being recommended [19].

Nonetheless, there are a number of pharmaceutical agents used in the treatment of T2DM with beneficial effects in NAFL, by targeting certain common pathophysiology mechanisms, such as insulin resistance.

## 3. Non-alcoholic fatty liver and SARS-COV2 infection

The disease caused by Coronavirus (COVID-19) was initially reported in the Wuhan Region, in China, in December 2019, and it represents a disorder caused by a newly identified viral agent, severe acute respiratory syndrome–related coronavirus 2 (SARS-COV2). Considering the high level of infectiousness and the risk of fast global spreading, on March 11, 2020 the World Health Organization declared the SARS-COV2 infection a global pandemic [3].

Thus, the scientific community has initiated global efforts to identify a number of risk factors contributing to a severe form of SARS-COV2 infection, the most important ones including the male gender, the presence of metabolic comorbidities, as well as T2DM and obesity or chronic liver diseases.

Liver impairment is frequently encountered among COVID-19 patients, being described in 16-53% of the cases, with a prevalence that reaches approximately 85% in the presence of the metabolic syndrome [20].

With regards to the liver injury risk factors, few studies have assessed this aspect thus far. A retrospective study assessing the liver damage of COVID-19 patients has described its greatest incidence among individuals with NAFL and a high BMI, while also identifying a string correlation between NAFL and the severe forms of SARS-COV2 infection [21]. Thus, NAFL, the hepatic consequence of the metabolic syndrome was included among the risk factors for an unfavorable prognosis [22].

The prognosis for NAFL associated with T2DM is given by the severity of the liver fibrosis that impacts not only the evolution of the SARS-COV2 infection, but also the vaccine response rate [23].

Thus, the assessment of liver fibrosis in the context of the COVID-19 pandemic exclusively relies on imaging techniques or non-invasive markers, and its assessment in the early stages of the infection can provide new information on the pathogenesis of the disease and could prove useful in stratifying the patient risks [24]. The FIB-4 score, a simple scoring system including AST, ALT, the age and thrombocyte count is the most researched non-invasive method for the assessment of liver fibrosis in the context of the SARS-COV2 infection [22, 25]. Nonetheless, studies have been carried with regards to the temporal dynamic of the score, being difficult to discern whether the increase of the FIB-4 values is caused by the underlying liver fibrosis or as a direct consequence of the cytopathic effect of the virus.

# II. Special part

## 1. Introduction

The non-alcoholic fatty liver (NAFL) diseases comprises a wide spectrum of clinical and histological entities that manifest through the progressive liver impairment.

NAFL currently has a global prevalence of 25.2%, with the highest rates in Southern America (31%) and the Middle East (32.5%), followed by Asia (27%), the United States of America (24%) and Europe (23%) [1]. Over the past two decades, the high prevalence of NAFL was accompanied by the progressive rise in the obesity and T2DM epidemics, disorders that coexist in most patients with metabolic dysfunctions.

Considering the increasingly strong evidence emphasizing T2DM as the most frequent cause of chronic liver disease [26, 27], the prognosis assessment and the clinical management of these patients needs to accurately indicate the status of the fibrosis, particularly of the severe fibrosis (F3). This subset of patients feature the highest risk of progression towards the advanced decompensated liver disease, NAFL-related hepatocarcinoma or even decease in the absence of a liver transplant.

NAFL has additionally demonstrated its impact on global health also through the negative effect on the COVID-19 evolution. Large population studies throughout the world have described metabolic disorders, particularly obesity and T2DM, as major risk factors for the severe form of COVID-19 [4]. This combination of metabolic dysfunctions, especially T2DM, represents a risk factor for MS-FL, especially since these comorbidities frequent coexist, the presence of NAFL leading to higher T2DM incidence rates, while T2DM accelerates the progression of NAFL towards advanced liver disease forms [28].

Current evidence shows that the presence of MS-FL is associated with the severe forms of COVID-19, the patients exhibiting a slower viral clearance and a higher probability of developing liver cytolysis during hospital stays [7].

## 2. Working hypothesis and general objectives

The arguments above underpinned the two studies comprised in this doctoral thesis, described in detail in the chapters to follow.

Considering the global NAFL and T2DM prevalence rate, approximately a quarter of the global population features a considerable risk of developing a severe form of COVID-19. Under the circumstances, the study **"The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on Covid-19 Disease Prognosis"** was initiated.

**The aim of the study** was to describe de liver impairment of a population of patients suffering from T2DM in the context of the SARS-COV2 infection and to assess the association of an increased FIB-4 score, as a liver fibrosis marker, with the prognosis of these patients.

**The objectives** of the study were:

- To describe the clinical and paraclinical parameters of a lot of T2DM patients in the context of the SARS-COV2 infection;
- To describe liver impairment in T2DM patients in the context of SARS-COV2 infection;
- To quantify liver steatosis and fibrosis among diabetic and COVID-19 patients;
- To identify the risk factors associated to an unfavorable COVID-19 prognosis.

The second study that is part of this doctoral thesis is entitles **"The Development of a Predictive Clinical Model for the Diagnosis of Severe Liver Fibrosis in Patients with Type 2 Diabetes Mellitus"** and it was initiated as part of the efforts to identify a non-invasive method for the assessment of liver fibrosis in T2DM patients, reducing the need for liver biopsy, with a particular focus on the identification of severe fibrosis (F>3).

**The aim of the study** was to review the performance of several non-invasive liver fibrosis assessment methods in a population of patients diagnosed with T2DM, and, subsequently, based on the results, to develop a predictive clinical model for the scoring of severe liver fibrosis.

**The objectives** of the study were:

- To describe the clinical and paraclinical parameters of the studied lot;
- To assess liver fibrosis using several non-invasive methods and to describe the lot in terms of the presence of severe fibrosis;

- To assess the performance of several parameters in the diagnosis of severe fibrosis (F>3);
- To develop a predictive clinical model for the diagnosis severe fibrosis (F>3).

## 3. General research methodology

Both studies have an analytical, prospective design, and included patients evaluated at the Gastroenterology Department of Bucharest University Emergency Hospital. The studies were approved by the Local Ethics Committee of Bucharest University Emergency Hospital (no. 9195/17 February 2021).

## 4. The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on Covid-19 Disease Prognosis

### 4.1 Introduction

The disease caused by Coronavirus 19 (COVID-19) is an infectious disorder secondary to a novel viral agent belonging to the Coroviridae family, i.e., severe acute respiratory syndrome–related coronavirus 2 (SARS-COV2), which mainly causes pulmonary impairment and that may progress towards the acute respiratory distress syndrome (ARDS) and exitus.

Most COVID-19 patients with metabolic syndrome develop liver impairment during the progression of the disease, most frequently in the case of severe SARS-COV2 forms [20]. Moreover, a retrospective study assessing the profiles of patients with liver damage has described its greatest incidence among individuals with NAFL and a high BMI, while also identifying a string correlation between NAFL and the severe forms of SARS-COV2 infection [21]. Thus, the NAFL syndrome was included among the risk factors for an unfavorable COVID-19 prognosis [22].

The prognosis for NAFL associated with T2DM is given by the severity of the liver fibrosis that impacts the evolution of the SARS-COV2 infection.

The information on the impact of hepatic fibrosis in the case of T2DM patients over the COVID-19 prognosis is still scarce. Hence, the aim of this study is to describe de liver impairment of a population of diabetic patients in the context of the SARS-COV2 infection and to assess the association of an increased FIB-4 score, as a liver fibrosis marker, with the prognosis of these patients.

## 4.2 Materials and methods

### 4.2.1 Study design

A prospective, analytical study was carried out, according Helsinki Declaration on the ethical Principles for medical research involving human subjects, which included 138 patients assessed at the Gastroenterology Department of Bucharest Emergency University Hospital.

### 4.2.2 Inclusion criteria

The inclusion criteria were:

- Age >18;
- Known type 2 diabetes mellitus diagnosed more than 6 months prior to the study start date, according to the criteria of the American Diabetes Association [29];
- Positive diagnosis of SARS-COV2 infection based on real-time reverse transcriptase-polymerase chain reaction (RT-PCR) performed on nasopharyngeal swabs.

### 4.2.3 Exclusion criteria

The exclusion criteria were:

- The presence of other liver disorder etiologies;
- The presence of myopathies;
- The presence of thrombocytopathies;
- The presence of ethanol consumption, defined as >20g/day for male subjects and as >30g/day for female subjects and assessed according to the AUDIT-C questionnaire recommended by the World Health Organization [30]

### 4.2.4 Patient assessment protocol

During the study, all the patients assessed at the Gastroenterology Department of Bucharest University Hospital were enrolled according to the criteria above. The enrolled subjects followed a specific protocol, which included anamnesis, the objective clinical examination and the paraclinical examinations.

#### 4.2.4.1 Anamnesis and clinical examination

Anamnesis and demographic data was collected at the baseline, including the age, gender, presence of comorbidities, the onset and duration of SARS-COV2 infection, the history and duration of pre-admission symptoms, the history of medication used, as well as weight and height measurements for each subject. Ethanol consumption was also assessed for each patient, according to the AUDIT-C questionnaire.

The objective clinical examination was carried out using devices and systems.

#### 4.2.4.2 Paraclinical examinations

Biological samples were collected for all patients, consisting of: hemogram, coagulation profile, liver function tests, renal function tests, lipid panel, serum iron, non-specific inflammatory tests, serum glucose, glycosylated hemoglobin and acid-base balance (ABB) profile.

For the diagnosis of SARS-COV2, a nasopharyngeal swab sample was collected, and the viral detection was performed via RT-PCR. The pulmonary damage was assessed through standard X-Ray scan and chest computed tomography (CT) scan.

#### 4.2.4.3 Liver steatosis and fibrosis assessment

The positive NAFL diagnosis was established through the exclusion, based on the anamnesis and on the paraclinical evidence, of other disorders that may lead to liver damage, as well as by reviewing the patients' history for evidence in this respect, i.e., imaging evidence and/or liver function test value changes over the past 12 months. Moreover, patients with a history of liver steatosis were assessed through the chest CT without contrast medium, the

protocol including the acquisition of hepatic slices at the level of the right portal vein and the superior splenic pole.

All NAFL patients were assessed for the presence of liver fibrosis, using the FIB-4 score [31]. Thus, depending on the scoring, patients were divided into 3 groups, using the standard cutoff values: low liver fibrosis risk patients (FIB-4<1.30), intermediate liver fibrosis risk patients (FIB-4: 1.30–3.25) and high liver fibrosis risk patients (FIB-4 > 3.25).

#### 4.2.4.2 Statistical analysis

All the data was collected and processed in Microsoft Excel, v. 2019. The statistical analysis was performed using Epi Info, v. 7.4.2.2020.

## 4.3 Results

### 4.3.1 General data of the study population

In this study, which included 138 diabetic patients diagnosed with COVID-19, the average age was of 66.32 ± 13.72, the lot comprising a higher number of male patients, i.e., 57.9% (n - 79), whereas women subjects represented 42.1% (n – 59) of the study population, with an average age of 66.32 ± 13.72. The average BMI was 29.91 ± 5.28.

NAFL was diagnosed in 91.3% (n - 126) of the cases, while 8.7% (n - 12) of the patients showed evidence of liver damage. According to the FIB-4 scoring, the patients were classified into three groups: 62.7% (*n* = 79) of the patients had a low liver fibrosis risk (FIB-4 < 1.30), 15.8% (*n* = 20) of the patients had an intermediate liver fibrosis risk (FIB-4: 1.30–3.25) and 21.5% (27) of the patients had a high liver fibrosis risk. (FIB-4 > 3.25) (figure 4.5).

The liver function test panel has shown that 73.9% (n – 102) of the patients had at least one altered parameter, most frequently LDH (81.5%). The damage patter most frequently was hepatocellular (64.6%), while the cholestasis enzymes increased in the advanced stages of the disease

### 4.3.2 Patient comparison based on the hepatic fibrosis status

The group of patients with a high liver fibrosis risk (FIB-4 > 3.25) had a higher BMI (31.3 ± 5.6 vs. 27.9 ± 5.2 vs. 27.2± 4.5, p<0.001) and higher ferritin values (690.57±197.85 vs. 625.87

± 201.24 vs. 623.45±198.56, p - 0.013), serum glucose values (198.25±87.68 vs. 164.54 ± 64.23 vs. 156.78± 65.24, p<0.001), glycosylated hemoglobin values ((7.8 (6.7 - 9.1) vs 7.2 (6.6-8.4) 0.037 vs. 7.1 (6.4 - 8.3), p – 0/037).

The liver function test values were statistically significantly higher in the group of patients with an increased risk of liver fibrosis. Thus, higher AST values have been observed ((57 (37 - 88) vs. 49 (36 -75) vs. 44 (29-85), p<0.001)), ALT ((47 (29 - 76) vs. 37 (21-62) vs. 35 (26-58), p<0.001)), GGT ((94 (45 - 123) vs. 72 (45 - 99) vs 69 (41- 102), p<0.001)), FAL ((79 (49 - 98) vs. 62 (42 - 91) vs. 53 (31 - 81), p<0.001)) and LDH ((391 (315-434) vs. 345 (215 - 357) vs. 317 (198 - 349), p - 0.002)).

Regarding the key parameters based on which the COVID-19 prognosis was assessed, the group of patients with a high risk of liver fibrosis (FIB-4 > 3.25) had a longer hospital stay (17 days vs. 13 days vs. 11 days, $p < 0.014$), a higher ICU admission rate (23% vs. 17.4% vs. 8.7%, $p = 0.021$) and a higher number of reported deaths (10.3% vs. 6.3% vs. 3.1%, $p < 0.001$). Moreover, the severe cases were more frequent among these patients (38.8% vs. 21.4%% vs. 9.5%, p - 0.019).

### 4.3.3 Risk factors associated to the unfavorable SARS-COV2 infection prognosis

The multivariate logistic regression analysis has identified the risk factors associated to the development of a severe form of COVID-19. The independent predictors were obesity (OR - 3.24; 95% CI, 1.46–5.32, $p = 0.003$), the high ferritin levels (OR - 1.9; 95% CI 1.78-8.29, $p = 0.031$) and the high FIB-4 score (OR-4.89; 95% CI, 1.34-12.3, $p = 0.02$).

## 4.4 Discussions

An important conclusion reached following this study was the association of high FIB-4 scores with the unfavorable COVID-19 evolution (hospital stay length, invasive ventilation need, rate of severe cases, as well as the number of deaths were statistically significantly higher). The outcomes are similar to those of *Campos-Murguia et al*, who carried out a retrospective study on a cohort of patients admitted for SARS-COV2 infection, to assess the effect of liver steatosis and fibrosis in the clinical spectrum of NAFL on the infection prognosis [32].

Under the circumstances, the hypothesis according to which the severe liver fibrosis accentuates the SARS-COV2-specific dysfunctional immune response may be regarded as valid.

As part of the independent review of the effect of each parameter over the SARS-COV2 infection prognosis, multivariate logistic regression analysis has shown that the presence of obesity (OR - 3.24, 95% CI, 1.46 - 5.32, *p* - 0.003), dyspnea (OR - 2.19, 95% CI, 1.56–6.29, p - 0.042), the high ferritin values (OR - 1.9, 95% CI, 1.78 – 8.29, *p* - 0.031) and the high FIB-4 score (OR - 4.89, 1.34 – 12.3, $p = 0.002$) lead to a higher risk of developing a severe form of COVID-19.

This study features certain limitations. The patient cohort included a Caucasian population of diabetic patients with a high prevalence of obesity and liver fibrosis. Another limitation concerns the use of the FIB-4 score. Nonetheless, considering the epidemiological risks of the SARS-COV infection that the medical professionals expose themselves to, the use of the FIB-4 was the safest alternative to minimize exposure, and the use of a different diagnostic method without biomarkers is quite difficult for the moment. Despite these limitations, our study still is one of the few scientific reports assessing the impact of higher FIB-4 scores in a population of diabetic patients over the SARS-COV2 infection prognosis.

### 4.5 Conclusions

In the group of patients with advanced fibrosis (FIB-4)-specific higher FIB-4 score values, the hospital stay length, the invasive ventilation need, the rate of severe cases, as well as the number of deaths were statistically significantly higher.

## 5. Clinical Model for the Prediction of Severe Liver Fibrosis in Adult Patients with Type II Diabetes Mellitus

### 5.1 Introduction

Non-alcoholic fatty liver (NAFL) comprises a wide spectrum of histological entities: simple liver steatosis (SS), non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis. Since it is characterized by a fatty load of >5% and by the exclusion of other liver damage

causes, NAFL coexists with metabolic disorders such as obesity or type 2 diabetes mellitus (T2DM).

The status of the liver fibrosis is the major advanced liver disease and liver complication-related predictor, an aspect that was noticed ever since its early stages.

Evan though liver biopsy is required for a correct diagnosis and staging, it has become increasingly seldom used and accepted by patients because of the potential complications.

Over the last decade several non-invasive methods have been developed to ascertain liver fibrosis. Thus, scoring scales validated on patient cohorts were developed, the most frequently used in the clinical practice being AST-to-platelet ratio index (APRI), Fibrosis 4 (FIB-4) or NAFLD fibrosis score (NFS).

Moreover, over the past years, the elastography techniques were developed for the assessment of liver fibrosis, in conjunction with ultrasound or not, the main advantage being that it is a quick, cost-efficient and non-invasive technique.

This study aims at assessing the diagnosis performance of several non-invasive liver fibrosis assessment methods in a population of diabetic subjects, using the concordance between the NFS score and the liver stiffness as measured through an ARFI method as reference. We have particularly focused on the severe fibrosis diagnosis, which represents the major liver progression trigger.

## 5.2 Materials and methods

### 5.2.1 Study design

A prospective, analytical study was carried out, according Helsinki Declaration on the ethical Principles for medical research involving human subjects, which included 175 patients assessed at the Gastroenterology Department of Bucharest Emergency University Hospital. Of them, 24 patients were excluded because of the erroneous pSWE measurements, and 17 were excluded because of the non-concordance between the pSWE and NFS score values. The study was approved by the Local Ethics Committee of Bucharest University Emergency Hospital (no. 9195/17 February 2021), and all enrolled patients have signed an informed consent form.

### 5.2.2 Inclusion criteria

The inclusion criteria were:

- Age >18;
- Known type 2 diabetes mellitus diagnosed more than 6 months prior to the study start date, according to the criteria of the American Diabetes Association [29];

### 5.2.3 Exclusion criteria

The exclusion criteria were:

- The presence of other liver disorder etiologies, i.e.:
- The presence of myopathies;
- The presence of thrombocytopathies;
- The presence of ethanol consumption, defined as >20g/day for male subjects and as >30g/day for female subjects and assessed according to the AUDIT-C questionnaire recommended by the World Health Organization [30]

### 5.2.4 Patient assessment protocol

#### 5.2.4.1 Anamnesis and clinical examination

Anamnesis and demographic data were collected at the baseline, including the age, gender, presence of comorbidities, the history of medication used, as well as weight and height measurements for each subject.

The objective clinical examination was carried out using devices and systems.

#### 5.2.4.2 Paraclinical examinations

Biological samples were collected for all patients, consisting of: hemogram, coagulation profile, liver function tests, renal function tests, lipid panel, serum iron, non-specific inflammatory tests, serum glucose, glycosylated hemoglobin, acid-base balance (ABB) profile, and serum insulin level. The insulin resistance was assessed according to the HOMA-IR score [33].

#### 5.2.4.3 Liver steatosis and fibrosis assessment

Liver steatosis and fibrosis were assessed using the Siemens Acuson S2000 ultrasound, v. VB20, Model no. 10041461 (Siemens Healthineers, 91052 Erlangen, Germany), with the 4C1 probe (4 MHz).

Module B was used to assess liver steatosis.

The liver fibrosis was assessed using the "Virtual Touch Tissue Quantification mode" software (Siemens Medical Solutions, Mountain View, CA, USA), integrated on the ultrasound device, according to the latest liver elastography recommendations of the European Society of Radiology [34].

Non-invasive biochemical-based marker scores were used to determine liver fibrosis. According to the current recommendations [35], the NFS, FIB-4 and APRI scores were used for each patient.

To define severe liver fibrosis, we have used a combined method consisting of the NFS score (>0.65) and pSWE value (>1.7 m/s) concordance rate, or the percentage of patients with protocol-compliant results for both tests (NFS>0.65 + pSWE >1.7 m/s). Thus, two groups of patients were defined: the group of F1/F2 fibrosis patients defined by a NFS score <0.65 and a pSWE value <1.7 m/s and the group of F3/F4 fibrosis patients, defined as a NFS score >0.65 and a pSWE value >1.7 m/s.

#### 5.2.4.4 Statistical analysis

The data was collected and processed in Microsoft Excel, v. 2019. The statistical analysis was carried using Epi Info v. 7.2.4.2020 and IBM-SPSS software v. 20.0.0.

## 5.3 Results

### 5.3.1 General data of the study population

The concordance rate between the NFS score and the pSWE value was of 88.7%, a total lot of 134 patients being enrolled for the final assessment. The average age was of 49.39 ± 8.19, the lot comprising a higher percentage of male subjects, i.e., 56.25% (n - 75), while female subjects represented 43.75% (n – 59).

The liver steatosis prevalence was of 85.8% (n - 115). Of them, 37.3% (n – 50) had first degree liver steatosis, 44% (n – 59) had second degree liver steatosis and 17.9% (n – 25) had third degree liver steatosis.

The prevalence of severe fibrosis (F3/F4) based on the NFS score and the pSWE value in the studied lot was of 18.7% (n – 25), while 81.3% (n – 109) of the patients were classified as exhibiting non-severe steatosis (F1/F2).

All patients enrolled were subjected to non-invasive tests for the assessment of liver fibrosis. The average values were: 1.3 ± 0.45 (m/s) for pSWE, NFS score - 0.29 (−1.5–2.4), FIB-4 - 2.74 (0.66–9.41), APRI - 1.02 ± 0.43 and Composite score - 1.6 (0-4).

### 5.3.2 Patient comparison based on the hepatic fibrosis status

The patients in group F3/F4 had advanced ages (55.41 ± 6.85 vs 48.66 ± 8.19, p<0.001), larger waist circumference (94.66 ± 12.64 vs. 91.96 ± 9.51, p - 0.053), as well as a higher BMI (30.22 ± 2.77 vs 28.11 ± 2.45, p – 0.02). Moreover, they featured a higher MS frequency (79.7% vs. 68.30%, p<0.001).

Regarding the laboratory test results, patients with severe liver fibrosis had higher levels of serum triglycerides (202.58 ± 54.92 vs 194.49 ± 60.7, p -0.032), AST (91.94 ± 16.3 vs 82.15 ± 18.14, p - 0.021), ALT (92.14 ± 23.13 vs 85.09 ± 31.9, p - 0.047) and GGT (140.02 ± 57.58 vs. 99.36 ± 48.76, p<0.001).

In so far as the non-invasive liver fibrosis assessment tests are concerned, patients with severe liver fibrosis had statistically significant higher NFS (1.48 (0.74–2.4) vs 0.01 (−1.5–1.8), p <0.001), FIB-4 (4.68 ± 1.95 vs 2.3 ± 0.74, p - 0.021), APRI (1.69 ± 0.5 vs 0.87 ± 0.2, p - 0.015) and pSWE scores (2.04 ± 0.26 vs 0.87 ± 0.22, p <0.001).

### 5.3.3 Diagnostic performance of the investigated tests

The age, the BMI, the GGT, the HOMA score, FIB-4, APRI and HbA1C were statistically significant predictors for the F3/F4 patient group. (AUROC 95% CI: 0.767 (0.627-0.907), 0.743 (0.578-0.908), 0.757 (0.617-0.897), 0.772 (0.616-0.924), 0.7 (0.548-0.852), 0.802 0.689-0.915), 0.791 (0.663-0.918), p<0.005 in all cases).

### 5.3.4 Severe liver fibrosis-related risk factors

Using the cutoff levels obtained pursuant to the ROC analysis, the GGT (>113 U/L), the age (>55 years of age), the BMI (>30.1 kg/m2), HOMA-IR (>3.3), HbA1C (>6.5%) and the Composite score represented independent predictors for the diagnosis of severe fibrosis in the researched lot (OR 95% CI: 8.993 (2.11-38.311), 7.453 (1.889-29.401), 5.996 (1.548-23.245), 5.879 (1.5413-22.431), 6.851 (1.954-19.547), 4.072(1.9-8.618), $p<0.05$ in all cases).

### 5.3.5 Severe fibrosis predictive clinical model established

According to the results of the multivariate analysis, we have developed a scoring system comprising the following parameters: GGT values <113 U/L and ≥113 U/L were quantified as 0 and 1, the age <55 and ≥55 was quantified as 0 and 1, the BMI <30.1 and ≥30.1 was quantified as 0 and 1, the HOMA-IR index <3.3 and ≥3.3 was quantified as 0 and 1, and HbA1C <6.5% and ≥6.5% was quantified as 0 and 1. Thus, the Composite score was defined as the sum of the GGT values, the age, the BMI, HOMA and HbA1C

The Composite score had the best diagnostic performance for the diagnosis of severe fibrosis ((0.899(0.792–0.986), $p < 0.005$), followed by the NFS, APRI and FIB-4 scores. Using a cutoff level of 3p, the sensitivity, specificity, the positive predictive value and the negative predictive value was of 85.3%, 91.2%, 79% and 89%, respectively.

## 5.4 Discussions

In the clinical spectrum of MS-FL, advanced fibrosis represents the most important prognosis marker, these patients featuring a high risk of progression towards decompensated cirrhosis and liver failure, as well as a high risk of developing NAFL-related hepatocarcinoma [36] [37].

In this study, advanced fibrosis (F3/F4) had a prevalence of 18.7%, with 81.3% of the patients included in the non-severe fibrosis group (F1/F2). Previous researches using biopsy as the reference method have described severe fibrosis prevalence rates similar to ours, of 16.2% to 43.1% [38, 39].

The biological profile of the researched cohort complies with the data obtained from studies carried out on large populations of diabetic subjects [40, 41].

In the assessed cohort, the multivariate regression analysis has shown that the GGT, age, BMI, HbA1C and HOMA-IR index values were independently associated with severe fibrosis. The results coincide with those of other studies in the literature, the association of severe fibrosis with metabolic syndrome elements being extensively documented [42, 43].

This study proposes a new clinical model for the prediction of severe fibrosis in diabetic patients, the Composite score, using parameters derived from the multivariate logistic regression analysis: GGT, age, BMI, HbA1C and the HOMA-IR index. The Composite score had the best diagnostic performance for the diagnosis of severe fibrosis ((0.899(0.792–0.986), $p < 0.005$), followed by the NFS, APRI and FIB-4 scores. Using a cutoff level of 3p, the sensitivity, specificity, the positive predictive value and the negative predictive value was of 85.3%, 91.2%, 79% and 89%

The study includes several strengths. The cohort was consecutively enrolled and described using demographic, clinical, biological and elastography-based parameters. We have particularly focused on documenting the diabetic status of the patients, which is a known risk factor in the progression of hepatic fibrosis. Following the scientific literature review on several search engines, this study is one of the first to assess a wide range of parameters and the link between them in a population at risk of developing advanced liver fibrosis, such as the diabetic one.

Nonetheless, the study protocol does feature some limitations. The assessed cohort was small in size, including a Caucasian population, with a high obesity and severe fibrosis prevalence. Another limitation of this study is related to the pSWE cutoff levels. The diagnostic performance of these values varies depending on the ultrasound systems used or on the liver disease etiology. Currently, there are no standard pSWE values in the case of NAFL patients, and insufficient evidence is available for the cutoff level >1.7m/s, future validation studies on larger populations being required.

### 5.5 Conclusions

In the case of T2DM patients, the BMI values, GGT, the advanced age, HbA1C and the HOMA-IR index represent independent predictors of advanced fibrosis. Including these clinical and laboratory parameters, the Composite score has had a good diagnostic performance in identifying diabetic patients at risk of developing severe fibrosis.

# 6. Conclusions and personal contributions

## 6.1 Final conclusions

This doctoral thesis comprises two studies:

- **"The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on Covid-19 Disease Prognosis"**
- **"The Development of a Predictive Clinical Model for the Diagnosis of Severe Liver Fibrosis in Patients with Type 2 Diabetes Mellitus"**

**The objectives of the first study** were:

- To describe the clinical and paraclinical parameters of a lot of T2DM patients in the presence of the SARS-COV2 infection;
- To describe liver impairment in T2DM patients in the presence of SARS-COV2 infection;
- To quantify liver steatosis and fibrosis, and to assess the patient lot based on the fibrosis status;
- To identify the risk factors associated to an unfavorable COVID-19 prognosis.

**The objectives of the second study** were:

- To describe the clinical and paraclinical parameters of the studied lot;
- To assess liver fibrosis using several non-invasive methods and to describe the lot in terms of the of severe fibrosis;
- To assess the performance of several parameters in the diagnosis of severe fibrosis (F>3);
- To develop a predictive clinical model for the diagnosis severe fibrosis (F>3).

I believe that all the objectives of this doctoral thesis have been reached, the conclusions reached being validated by the results in the literature.

### 6.2 Personal contributions

The main purpose of this doctoral thesis was to identify a non-invasive standard of care for the determination of advance liver fibrosis in T2DM patients. Thus, following the review of the literature, two papers were drafted, **"The severity of non-alcoholic fatty liver disease in type II diabetes"** [44] and "**Non-alcoholic fatty liver disease in diabetic patients as risk factor for poor prognosis of covid-19: an update of potential mechanisms and treatment considerations**" [7], where, together with my collaborators, I have summarized the main pathophysiological relations between these diseases, as well as current management notions. Furthermore, my personal contribution materializes, in the case of the first study representing the subject of this thesis -"**The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on Covid-19 Disease Prognosis"** [45]- through the demonstration of the relation between advanced fibrosis and the unfavorable diseases progression, as well as through the identification of independent predictors of the poor prognosis of the SARS-COV2 infection, advanced fibrosis being one of them. I believe that this data importantly contributes to the stratification of the risk in SARS-COV2 patients and may help reduce medical care-related costs, especially in areas with a high metabolic disorder prevalence. Subsequently, my personal contribution also materialized in the second study – **"The Development of a Predictive Clinical Model for the Diagnosis of Severe Liver Fibrosis in Patients with Type 2 Diabetes Mellitus" -** through the development of a safe, widely available, reproducible non-invasive method for the assessment of severe liver fibrosis in diabetic patients, with a potential of reducing the use of liver biopsy.

Last but not least, my personal contribution also consisted of covering all the (steep) stages towards the achievement of the final goal of this doctoral thesis, starting from the development of the study methodology and of the patient assessment protocol. Despite the onset of the COVID-19 shortly after, a time of distress for the society but especially for the medical system, it has been shown that limits are there only to be overcome, especially in the healthcare system. Thus, in the context of the well-known epidemiological challenges, I have continued to enroll patients and perform the clinical and paraclinical investigations, followed by the collection and processing of information and finally, after extensive work and documentation, I have managed to explain and statistically analyze the data.

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# Appendix

**List of articles published within the doctoral research:**

- **From the topic of the doctoral thesis**

- **Calapod OP**, Marin AM, Pantea Stoian A, Fierbinteanu-Braticevici C. Clinical Model for the Prediction of Severe Liver Fibrosis in Adult Patients with Type II Diabetes Mellitus. Diagnostics (Basel). 2022 Jul 29;12(8):1829. doi: 10.3390/diagnostics12081829. PMID: 36010180; PMCID: PMC9406388. ISI - **IF – 3,992**
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- **Calapod OP**, Marin AM, Onisai M, Tribus LC, Pop CS, Fierbinteanu-Braticevici C. The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on Covid-19 Disease Prognosis. Medicina (Kaunas). 2021 Apr 30;57(5):434. doi: 10.3390/medicina57050434. PMID: 33946377; PMCID: PMC8147130. **ISI – IF:2.43**
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- **Calapod OP**, Marin, A.M.; Tribus, L.C.; Fierbinjeanu-Braticevici, C. Non-alcoholic fatty liver disease in diabetic patients as risk factor for poor prognosis of covid-19: An update of potential mechanisms and treatment considerations. Farmacia 2020, 68, 785–789. **ISI - IF: 1,443**
  https://farmaciajournal.com/issue-articles/non-alcoholic-fatty-liver-disease-in-diabetic-patients-as-risk-factor-for-poor-prognosis-of-covid-19-an-update-of-potential-mechanisms-and-treatment-considerations/

- **Calapod, Ovidiu Paul**, Marin, Andreea Maria, Tribus, Laura Carina and Fierbinţeanu-Braticevici, Carmen. "The Severity of Non-Alcoholic Fatty Liver in Type II Diabetes" Internal Medicine, vol.15, no.6, 2019, pp.37-42. **BDI**
  https://doi.org/10.2478/inmed-2018-0044.

- **Ovidiu P Calapod**, Andreea M Marin, Minodora Onisai, Laura C Tribus, Corina S Pop, Carmen Fierbinteanu-Braticevici. The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on COVID-19 Disease Prognosis. In: P Syamasundar Rao, editor. Prime Archives in Medicine: 4th Edition. Hyderabad, India: Vide Leaf. 2022. **BOOK CHAPTER**

- **With a topic different from the doctoral thesis**

- Marin AM, **Calapod OP**, Moldoveanu AC, Tribus LC, Fierbințeanu-Braticevici C. Non-invasive Ultrasonographic Score for Assessment of the Severity of Inflammatory Bowel Disease. Ultrasound Med Biol. 2021 Apr;47(4):932-940. doi: 10.1016/j.ultrasmedbio.2020.11.026. Epub 2020 Dec 30. PMID: 33388210. **ISI - IF: 2,514** https://www.umbjournal.org/article/S0301-5629(20)30535-4/fulltext

- Minodora Onisai, Ana Maria Vlădăreanu, Iuliana Iordan, Cristina Enache, **Ovidiu Calapod**, Andreea Marin, Carmen Georgeta Fierbinţeanu-Braticevici, Laura Tribus. "Hematological alterations in hepatic cirrhosis – a challenge in clinical practice", Oncolog Hematolog, No. 54 (1) 2021 • DOI: 10.26416/OnHe.54.1.2021. **BDI** https://medichub.ro/reviste-de-specialitate/oncolog-hematolog-ro/modificari-hematologice-in-ciroza-hepatica-o-provocare-in-practica-clinica-id-4575-cmsid-68

- Laura Tribus, Carmen Georgeta Fierbinţeanu-Braticevici, Andreea Marin, **Ovidiu Calapod**, Ana Maria Vlădăreanu, Horia Bumbea, Iuliana Iordan, Minodora Onisai. "Rare disease with atypical onset and multidisciplinary approach", Oncolog Hematolog, No. 54 (1) 2021 • DOI: 10.26416/OnHe.54.1.2021. **BDI** https://medichub.ro/reviste-de-specialitate/oncolog-hematolog-ro/patologie-rara-cu-debut-atipic-si-abordare-multidisciplinara-id-4577-cmsid-68

- **Calapod, Ovidiu Paul**, Marin, Andreea Maria, Tribus, Laura Carina and Fierbinţeanu-Braticevici, Carmen. "Sarcopenia in cirrhosis: a systematic review" Romanian Journal of Orthopaedic Surgery and Traumatology, vol.2, no.2, 2019, pp.125-129. **BDI** https://doi.org/10.2478/rojost-2019-0023

**Papers presented at scientific events**

- "An unusual case of fulminant ulcerative colitis in a 77 years old patient" Andreea Maria Marin, **Ovidiu Paul Calapod**, Laura Carina Tribus, Carmen Fierbinteanu-Braticevici, Al 38-lea Congres National de Gastroenterologie, Hepatologie si Endoscopie Digestiva, 2018; **POSTER**
- "The use of blood and fecal biomarkers to evaluate the endoscopic activity of ulcerative colitis" **Ovidiu Paul Calapod**, Andreea Maria Marin, Laura Carina Tribus, Carmen Fierbinteanu-Braticevici, Al 38-lea Congres National de Gastroenterologie, Hepatologie si Endoscopie Digestiva, 2018; **POSTER**
- "Hereditary hemochromatosis: case study in a patient with diabetes mellitus and restrictive cardiomyopathy" **Ovidiu Paul Calapod**, Andreea Maria Marin, Laura Carina Tribus, Carmen Fierbinteanu-Braticevici, Al 38-lea Congres National de Gastroenterologie, Hepatologie si Endoscopie Digestiva, 2018; **POSTER**
- "The Impact of Increased Fib-4 Score in Patients with Type II Diabetes Mellitus on Covid-19 Disease Prognosis" **Calapod, Ovidiu Paul**, Marin, Andreea Maria, Necula, Ana, Fierbinţeanu-Braticevici, Carmen, Al 41-lea Congres National de Gastroenterologie, Hepatologie si Endoscopie Digestiva, 2022; **POSTER**
- "Giant pseudoanevrysm of the splenic artery", Actualitati in Gastroenterologie, 2022; **ORAL PRESENTATION**
- "Complex CBD stones: know your tools", Gastromaraton, 2021; **PREZENTARE ORALĂ**
- "A rare complication in chronic pancreatitis", Raport de garda intre specialitati, 13-14 noimebrie, 2020; **ORAL PRESENTATION**
- Icter egal ciroza?", Raport de garda intre specialiati, 15-16 martie 2019; **ORAL PRESENTATION**
- "What lies beneath", Actualități in Gastroenterologie, 29-30 septembrie 2018; **ORAL PRESENTATION**