GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

CHANGING THE NATURAL HISTORY OF ADVANCED LIVER AND GASTROINTESTINAL DISEASES

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The task of the excellent teacher is to stimulate 'apparently ordinary' people to unusual effort. The tough problem is not in identifying winners: it is in making winners out of ordinary people.

K. Patricia Cross

CONTENTS

ABBREVIATION LIST

AASLD - American Association for the Study of Liver Diseases
ACLF - acute-on-chronic liver failure
AFP - alpha fetoprotein
AKI - acute kidney injury
ALD - alcoholic liver disease
APASL - Asian Pacific Association for the Study of the Liver
APRI - aspartate aminotransferase to platelet ratio index
BMI - body mass index
BMD - bone mineral density
CAP - controlled attenuation parameter
CD - Crohn's disease
C. difficile - *Clostridium difficile/ Clostridioides difficile*
CDI - *Clostridium difficile* colitis
CLD - chronic liver disease
CLIF-C OF - Chronic Liver Failure Consortium Organ Failure
CRP - C-reactive protein
C282Y, H63D - mutations in the HFE gene
DAA - direct-acting antiviral therapy
EASL - The European Association for the Study of the Liver
EOT - end of treatment
FDA - Food and Drug Administration
FIB-4 - fibrosis-4 index
GPx - glutathione peroxidase
HBV - chronic hepatitis B virus
HCC - hepatocellular carcinoma
HCV - chronic hepatitis C virus
HE - hepatic encephalopathy
HFE - the hereditary hemochromatosis gene
HHC - hereditary hemochromatosis
HJV - the genes encoding hemojuvelin
HVPG - hepatic venous pressure gradient
IBD - inflammatory bowel disease
IFN - interferon
INR - international normalized ratio
LB - liver biopsy
LC - liver cirrhosis
LED/SOF - Ledipasvir/Sofosbuvir
LF - liver fibrosis
LSM - liver stiffness measurement
LT - liver transplantation
MDA - malondialdehyde
METAVIR - meta-analysis of histological data in viral hepatitis
MHE - minimal hepatic (covert) encephalopathy
MELD - Model of End-Stage Liver Disease
MVP - mean platelet volume

NAFLD - non-alcoholic fatty liver disease
NAP1 - North-American Pulsed-Field Type 1
NASH - non-alcoholic steatohepatitis
NFS - NAFLD fibrosis score
NSBBs - nonselective beta-blockers
OGC - oral glutamine challenge
OHE - overt hepatic encephalopathy
PCR - polymerase chain reaction
PCT - plateletcrit
PCR-RFLP - polymerase chain reaction-restriction fragment length polymorphism
PPI - proton pump inhibitors
PrOD - Paritaprevir/Ritonavir, Ombitasvir and Dasabuvir
PT - prothrombin time
PVT - portal vein thrombosis
QT - Quick time
RBV - Ribavirin
ROS - reactive oxygen species
SARS CoV2 - severe acute respiratory syndrome coronavirus 2
SBP - spontaneous bacterial peritonitis
SD - standard deviation
SLC40A1, SLC11A3 - the genes encoding ferroportin
SOD - superoxide dismutase activity
SOFA - sequential organ failure assessment
SRGH - Romanian Society of Gastroenterology and Hepatology
SVR - sustained virological response
T2DM - diabetes mellitus
TE - transient elastography
UC - ulcerative colitis
VA - venous ammonia
VB - variceal bleeding
VCTE - vibration-controlled transient elastography
WHO - World Health Organization
WtHR - waist-to-height ratio

REZUMAT

Teza de abilitare "**Changing the Natural History of Advanced Liver and Gastrointestinal Diseases**" reprezintă o sinteză a activității mele profesionale, didactice și de cercetare științifică desfășurată în perioada postdoctorală.

Conform recomandărilor Consiliului Național de Atestare a Titlurilor, Diplomelor Certificatelor Universitare (CNADTCU) teza de abilitare este structurată pe trei secțiuni:

- Secțiunea I – Realizări științifice din perioada postdoctorală.
- Secțiunea II – Proiecte viitoare în activitatea profesională, academică și științifică.
- Secțiunea III – Referințe.

Secțiunea I debutează cu o scurtă trecere în revistă a activităților mele profesionale, academice și științifice. Am evidențiat principalele elemente ale activității clinice și academice, activități care se întrepătrund și se desfășoară în același timp, la capul pacientului. În introducere sunt sintetizate realizările științifice concretizate în: 57 capitole de carte (45 dintre ele elaborate în perioada postdoctorală) publicate în cărți medicale de specialitate, tratate medicale, și una dintre ele în editură internațională; 44 articole publicate în extenso în reviste indexate ISI cu factor de impact (14 ca autor principal și 30 co-autor);41 de articole publicate în reviste ISI Proceedings și reviste indexate BDI.

De asemenea, este prezentată lista cu lucrările științifice premiate de societăți profesionale naționale (14) și internaționale (4).

Recunoașterea activității științifice este dovedită de:

- indicele Hirsh - 11 (WOS),
- 342 citări și un factor de impact cumulat (pentru articolele în care sunt autor principal) de 31.393.

Menționez că am participat cu numeroase comunicări orale și postere la manifestări naționale și internaționale și am peste 100 de rezumate publicate în suplimente ale unor reviste indexate ISI și în volumele unor manifestări științifice cu ISBN. Pe parcursul lucrării am încercat să evidențiez valoarea științifică deosebită a unora dintre temele de cercetare, acestea fiind abordate și publicate pentru prima dată în România.

Am detaliat contribuțiile în direcțiile de cercetare abordate de la momentul obținerii titlului de doctor în medicină și până în prezent, incluzând totodată principalele idei și rezultate din articole relevante din activitatea mea științifică. Rezultatele obținute au fost concretizate prin publicarea de articole indexate Thomson ISI Web of Science Core Collection, iar unele sunt indexate în baze de date internaționale.

Cea mai importantă parte a secțiunii, și de fapt a tezei, o constituie prezentarea rezultatelor activității științifice. Prin expunerea si interpretarea rezultatelor cercetării științifice, am dorit să subliniez importanța cunoașterii istoriei naturale a bolii hepatice/gastroenterologice și, totodată, posibile intervenții profilactice și terapeutice care pot influența prognosticul pacientului cu status critic, profil frecvent întâlnit în activitatea mea.

Temele de cercetare sunt organizate în trei capitole, pe parcursul cărora am prezentat date teoretice detaliate în cadrul stadiului actual al cunoașterii în domeniu, rezultatele obținute în urma cercetărilor proprii, punctând elementele de originalitate și de noutate în România.

Tema principală abordată în lucrarea de față este reprezentată de pacientul cu boală cronică hepatică avansată, afecțiune ce predomină în cadrul activității mele clinice. Scopul cercetării științifice este reprezentat de stabilirea cauzelor care au dus la creșterea frecvenței acestei afecțiuni, și de asemenea, am încercat să evaluez posibilitățile terapeutice ce ar putea fi abordate pentru reducerea morbidității și mortalității la pacienții cu boală hepatică avansată.

Primul capitol abordează ca direcție principală de cercetare etiologia bolii cronice hepatice. Importanța stabilirii factorilor etiologici ai bolii hepatice reprezintă o preocupare permanentă a cercetătorilor din domeniul hepatologiei, boala cronică hepatică fiind o problemă

majoră de sănătate publică la nivel mondial. În această direcție, menționez că am publicat *primele date referitoare la mutațiile hemocromatozei ereditare în România***.** Pe parcursul acestui capitol, am evaluat etiologia și incidența bolii cronice hepatice cu scopul de a stabili unde și cum putem interveni pentru a reduce numărul cazurilor nou-diagnosticate cu ciroză hepatică. De asemenea, prin prezentarea celor mai actuale date teoretice disponibile în literatura de specialitate, precum și prin expunerea sistematizată a rezultatelor cercetării, am argumentat importanța și avantajele utilizării metodelor non-invazive de evaluare a bolii cronice hepatice.

Al doilea capitol al acestei secțiuni abordează problematica pacientului cu boală hepatică cronică avansată și complicațiile asociate bolii. Pe parcursul capitolului am prezentat o sinteză a celor mai recente date existente în literatura medicală științifică referitoare la boala hepatica cronică, și am prezentat rezultate originale obținute în urma cercetării. În acest context, trebuie să subliniez că *am implementat în România tratamentul standardizat al hemoragiei variceale conform ghidurilor internaționale*. Tot în acest capitol principalele complicații ale bolii hepatice avansate cu scopul de a aprecia ce putem face pentru acești pacienți pentru a le modifica și ameliora evoluția bolii.

Al treilea capitol al cercetării abordează o patologie de actualitate, colita cu *Clostridium difficile*. Această afecțiune este de mare interes pentru comunitatea științifică, fiind un subiect intens cercetat în ultimii ani, creșterea preocupării cercetătorilor din domeniul gastroenterologiei pentru acest subiect fiind obiectivată de multitudinea de studii publicate în literatura medicală internațională. Expertiza noastră în această patologie este confirmată de articolele publicate în literatura de specialitate. Cercetarea în acest domeniu a abordat spectrul patologic al colitei cu *Clostridium difficile* și intervențiile terapeutice la principalele grupe de risc precum pacienții cu boli inflamatorii intestinale, ciroză hepatică, vârstnici și, de mare actualitate, la pacienții cu infecție SARS-CoV2. Trebuie să subliniez că *am publicat primul caz de infecție cu Clostridium difficile în România* și, totodată, că am fost primii care am încercat să creștem conștientizarea problematicii atât în cadrul personalului medical, cât și la nivel populațional.

Secțiunea II prezintă proiectele viitoare în domeniul clinic, academic și științific; Această secțiune include descrierea detaliată a activității științifice, obiectivul tezei de abilitare. Experiența acumulată, precum și realizările științifice din perioada post-doctorală, reprezintă argumente pentru formularea obiectivelor și strategiilor de dezvoltare pe plan didactic, profesional și științific. În concordanță cu activitatea clinică (managementul pacientului critic, cu boală hepatică avansată), temele principale de cercetare vor avea ca scop modificarea istoriei naturale și ameliorarea prognosticului pacienților cu boală cronică hepatică prin creșterea supraviețuirii și creșterea calității vieții. Din moment ce nu avem terapii specifice eficiente pentru modificarea evoluției pacienților cu boală hepatică decompensată proiectele pe termen scurt vor încerca să găsească noi valențe ale medicamentelor utilizate de zeci de ani în tratamentul bolilor cronice hepatice. De asemenea, îmi propun să studiez corelarea metodelor noninvazive de evaluare a fibrozei și steatozei hepatice în alte situații decât cele studiate până acum.

Secțiunea III include referințele bibliografice relevante pentru subiectele discutate, care au fost utilizate pentru elaborarea acestei teze și pentru articolele publicate pe parcursul carierei științifice. Un număr considerabil de referințe sunt de actualitate, din ultimii 5 ani, ceea ce demonstrează că subiectul și temele din teza mea sunt de actualitate și intens studiate de comunitatea științifică, și continuă să reprezinte provocări în activitatea clinică și de cercetare.

Teza de abilitare prezintă sinteza activității mele postdoctorale, fiind totodată o realizare comună a colectivului de excepție din care fac parte.

Validarea tezei reprezintă un argument pentru confirmarea rezultatelor noastre, obținerea dreptului de îndrumare a doctoranzilor, precum și suport pentru proiectele viitoare.

ABSTRACT OF THE THESIS

The habilitation thesis "**Changing the Natural History of Advanced Liver and Gastrointestinal Diseases"** is a synthesis of my professional, didactic and scientific research activity carried out in the postdoctoral period. According to the recommendations of the National Council for Attestation of Degrees, Diplomas and University Certificates (CNADTCU) the thesis is structured on three sections:

- Section I - Scientific achievements from the postdoctoral period.
- Section II - Future projects in professional, academic and scientific activity.
- Section III - References.

In **Section I**, I present a brief overview of my professional, academic, and scientific activities. We have pointed out the main elements of the clinical and academic activity, activities that are connected and take place at the same time, next to the patient's bed.

The introduction summarizes the scientific achievements embodied in: 57 book chapters (45 of them in the postdoctoral period) published in specialized medical books, medical treatises and one of them in international publishing, 44 articles published extensively in ISI indexed journals with impact factor (14 lead author and 30 co-author), 41 articles published in ISI Proceedings and BDI indexed journals.

The list of scientific papers awarded by national (14) or international professional societies (4) is also presented.

The recognition of scientific activity is proven by:

- the Hirsh index of 11 (WOS)
- 342 citations with a cumulative impact factor (for the articles in which I am the principal author) of 31.393.

I have participated with numerous oral communications and posters in national and international events and I have over 100 abstracts published in supplements of ISI indexed journals and in the volumes of scientific events with ISBN. In this paper, I tried to highlight the scientific value of some of the research topics, some of these being addressed and published for the first time in Romania.

I have detailed the contributions in the research directions approached from the moment of obtaining the title of doctor in medicine and until now, including at the same time the main ideas and results from relevant articles in my scientific activity. The results were materialized by publishing indexed articles Thomson ISI Web of Science Core Collection, and some are indexed in international databases.

The most important part of the section, and in fact the thesis, is the presentation of the results of the scientific activity approached. By presenting and interpreting the results of scientific research, I wanted to emphasize the importance of knowing the natural history of liver/gastroenterological disease and also possible prophylactic and therapeutic interventions that can influence the prognosis of critically ill patients, a common profile in my work.

The research topics are organized in three chapters, during which we presented detailed theoretical data in the current state of knowledge in the field, the results obtained from our own research, pointing out the elements of originality and novelty in Romania.

The main topic addressed in this paper is the patient with advanced chronic liver disease, a condition that predominates in my clinical work. The purpose of my scientific research is to determine the causes that have led to an increase in the incidence of this condition, and I have also tried to evaluate the therapeutic possibilities that could be addressed to reduce morbidity and mortality in patients with advanced liver disease.

The first chapter focuses on the etiology of chronic liver disease. The importance of determining the etiological factors of liver disease is a permanent concern of researchers in the field of hepatology, being a major public health issue worldwide. In this direction, I

mention that I have *published the first data regarding the mutations of hereditary hemochromatosis in Romania.* During this chapter, I have evaluated the etiology and incidence of chronic liver disease in order to determine where and how we can intervene to reduce the number of newly diagnosed cases of liver cirrhosis. Also, by presenting the most current theoretical data available in the literature, as well as by systematically presenting the results of research, we argued the importance and benefits of using non-invasive methods for assessing chronic liver disease.

The second chapter of this section addresses the issue of the patient with advanced chronic liver disease and the complications associated with the disease. Throughout the chapter, I have presented a summary of the latest data in the scientific medical literature on chronic liver disease, and I have presented original results obtained from the research. In this context, I must emphasize that *we have implemented in Romania the standardized treatment of variceal hemorrhage according to international guidelines*. At the same time, during the research activity, I analyzed the main complications of advanced liver disease in order to evaluate what we can do for these patients to modify and improve the evolution of the disease.

The third chapter of the research addresses a current pathology, *Clostridium difficile* colitis. This condition is of great interest to the scientific community, being an intensely researched topic in recent years, the growing concern of researchers in the field of gastroenterology for this topic being demonstrated by the multitude of studies published in the international medical literature. Our expertise in this disease is confirmed by the articles we have published in the literature. My research in this area addresses the pathological spectrum of *Clostridium difficile* colitis and therapeutic interventions in major risk groups such as patients with inflammatory bowel disease, liver cirrhosis, the elderly and, most recently, patients with SARS-CoV2 infection. I must emphasize that *we published the first case of Clostridium difficile infection in Romania* and, at the same time, that we were the first to try to raise awareness of the issue both among the medical staff and at the population level.

Section II presents my future projects in the clinical, academic and scientific fields. This section includes a detailed description of the scientific activity, the objective of the habilitation thesis. Experience and achievements in the post-doctoral period are arguments for formulating didactic, professional and scientific development objectives and strategies. Consistent with the clinical activity (critical patient management, with advanced liver disease), the main research topics will aim to change the natural history and improve the prognosis of these patients by increasing survival and increasing quality of life. Since we do not have specific effective therapies to change the course of patients with decompensated liver disease, short-term projects will try to find new valences of drugs used for decades in the treatment of chronic liver disease. I also intend to study the correlation of noninvasive methods for assessing liver fibrosis and steatosis in other situations than those studied so far.

Section III includes the relevant bibliographical references for the topics discussed, which were used for the elaboration of this thesis and for the articles published during the scientific career. A considerable number of references are current, from the last 5 years, which proves that the subject and themes of my thesis are real and intensively studied by the scientific community, and that they continue to represent challenges in the clinical activity.

The habilitation thesis presents the synthesis of my postdoctoral activity, being at the same time a common achievement of the exceptional team of which I am part.

The validation of the thesis is an argument for confirming our results, obtaining the right to guide doctoral studies, as well as support for future projects.

SECTION I

BACKGROUND OF PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACCOMPLISHMENTS

A. OVERVIEW OF THE ACHIEVEMENTS TO DATE

This thesis represents the synthesis of my entire activity - clinical, scientific and academic. According to the Explanatory Dictionary of the Romanian Language, ability means "Giving someone a certain title, a degree, etc., giving them the right to practice a certain profession."

I believe that I have reached professional maturity and the qualification would allow me to continue to offer what I acknowledged and received from my mentors; as long as they guided me and gave me the chance to develop professionally, I feel obliged to continue and to guide and give forward everything I can.

Any medical school graduate who has the chance of a teaching career must be aware that his activity must be dynamic and in continuous improvement. I knew from the beginning of my university teaching career that I would have to progress and continually develop my professional activities.

I have the chance to practice two of the most beautiful, noble, and humanistic professions (medicine and education). These professions require dedication, involvement, compassion; both of them require a lot of professional consumption and emotional involvement but also bring enormous satisfaction.

Our academic environment offers many opportunities and a multitude of duties. I am an associate teacher (a profession that involves teaching and research), a specialist doctor working in a University Hospital (a career that involves clinical activity) and I intent to continuously evolve in all three directions: clinical, teaching, and research. All these directions cannot be analyzed and evaluated individually because my didactic, clinical, and research activities are connected and carried out somewhat simultaneously.

1. CLINICAL ACTIVITY

My primary training is as an internist, the most complex medical specialty, which integrates all medical knowledge and solves complex pathologies. The chance to work in a competitive team (the former II-nd Medical Clinic, current Institute of Gastroenterology and Hepatology) and the dedication to patients were continuous challenges to improve and develop my activity. The advantage of internal medicine is collaboration and semiology.

My internship in internal medicine within the residency program carried out under the guidance of Prof. Dr. Carol Stanciu and Anca Trifan was the moment that marked my entire subsequent professional evolution, the achievements in clinically, teaching, and research activity.

I had the chance to compete for the position of assistant professor in medical semiology, to carry out doctoral studies, and to pass the exam for another generous specialty (gastroenterology), which allowed me to work in an elite institution of Romanian gastroenterology - Institute of Gastroenterology and Hepatology Iasi (IGH).

Shortly after the start of my internship in internal medicine, my clinical activity was constantly and harmoniously carried out with the didactic and research activity.

Along my 30 years of clinical practice, I've gone through all the stages of the medical career: physician specialist (internal medicine and gastroenterology), physician consultant

(internal medicine and gastroenterology). Over time I have acquired skills and competencies in general ultrasonography, non-invasive evaluation of liver fibrosis, diagnostic, and therapeutic digestive endoscopy; I have advanced training courses in Elastometry internship by Hitachi – Craiova, 2007 and Intensive Contrast-Enhanced Ultrasound Course, 2010, Timisoara. In 2001 I participated in a training course in the Netherlands, in Zwolle and Arnhem hospitals.

During all these 30 years, I provided competent medical assistance (emergency, out and in-patients with specific gastroenterological pathology) in a tertiary reference center for Moldova; I performed thousands of hours of on-call duties in gastroenterology, investigations (abdominal ultrasounds, diagnostic and therapeutic upper and lower digestive endoscopes, etc.).

Since the establishment of the Institute of Gastroenterology and Hepatology, I have worked mainly in the **acute therapy unit** and provided medical assistance for major gastroenterological emergencies. As the primary pathology of the Institute is chronic liver disease, most patients from the critical therapy sector are those with major complications of liver cirrhosis (upper gastrointestinal bleeding, liver failure, etc.). In this context, under the guidance of Prof. Carol Stanciu, we implemented for the first time in Romania the **standardized treatment of acute variceal bleeding** in cirrhotic patients, according to international guidelines - a personal model. The constant application of the protocol following the Baveno consensus has brought us professional satisfaction, namely, the reduction over the years of mortality by variceal bleeding in cirrhotic patients.

Throughout my activity in this service, I have educated the average medical and care staff to provide medical care to patients with gastroenterological and hepatic emergencies and I managed to form a competent team dedicated to specific pathologies in the acute therapy unit. Our efforts have resulted in a reduction in mortality from variceal bleeding from 40% before 2000 to about 20% in recent years.

Most of our patients are patients with liver cirrhosis (often with advanced liver disease), and the ideal treatment for them is **liver transplantation**. I am pleased to remember, and I want to emphasize that more than 14 years ago, our team coordinated by Prof. Anca Trifan at the initiative of Prof. Carol Stanciu activated for the inclusion of our university center in the liver transplant program. Our activity (implementation of criteria for selecting potential donors, awareness of doctors, population, civil society, etc.) was challenging at that time; pioneering in Moldova was materialized by a brochure - a practical guide for evaluating potential donors. Although our work seemed to be in vain at that time, we are sure that our effort was an essential step for the inclusion of Iasi in 2016 on the map of liver transplant centers in Romania. Iasi is the only liver transplant center outside Bucharest and its recognition has proved especially beneficial for patients from Moldovian area.

In 2011 we have published the **first case of CDI in Romania**, in a 72-year old man with diarrhea, hypoproteinemia, edema and ascites (*J Gastrointestin Liver Dis* 2011). I would like to emphasize our contribution to raising awareness about CDI because most cases were underdiagnosed because of low levels of awareness for CDI among clinicians.

In 2007 I attended the hospital management course (no.199/ National School of Public Health and Health Management: MS3/1876). Between 2007 -2008 I was the **medical director** (admitted by competition) of the Institute of Gastroenterology and Hepatology Iași (legal personality). Our team has contributed substantially to designing a modern sanitary unit according to international standards.

My professional career was built within the team where I work; development, continuous personal improvement and close collaboration with colleagues are the essential elements for a successful career, with both individual and team satisfaction.

2. ACADEMIC ACTIVITY

My teaching career began in 2000 as an Assistant Professor in Medical Semiology at "Grigore T. Popa" University of Medicine and Pharmacy - position obtained after a contest. Since 2009 I have been Lector in the discipline Medical Semiology-Gastroenterology, and since 2018 I have been Associate Professor in the same discipline. During this period I held medical semiology internships with 3rd year students of the Faculty of Medicine, internships and gastroenterology courses with 5th year students of the Faculty of Medicine. Following the directions of the University of Medicine and Pharmacy "Grigore T. Popa" Iasi, I was also tutor at the College of Nurses, Botosani and Iasi divisions. Most of the teaching activity took place in the series of students in Romanian language series, but we also had groups of students from the series taught in English language.

In all my activities, I have consistently demonstrated the availability of work in all directions (teaching, clinical, research).

For over ten years, I have been teaching (initially as a collaborator of Prof. Dr. Anca Trifan then as a course holder) the **elective course** of digestive endoscopy for fifth-year general medicine students, a course that enjoys great interest among students (over 60 students/year). With each course I hold I am trying to make them more appealing (through workshops, lectures, and practical internships on the simulator) and interactive. My goal is to make students participate actively, not just listen to a course and also to develop their decision-making capacity in cases with borderline situations.

I was and am actively involved in the **postgraduate course** for obtaining competence in diagnostic digestive endoscopy coordinated by Prof. Anca Trifan. I participate both in the practical and theoretical preparation of the participants and in the organization and support of the final exam.

My teaching activity also consists of training gastroenterology fellows. I provided them guidance for their daily duties, courses and internships. I guided them in the medical care provided to patients in emergencies and coordinated digestive endoscopic examinations. All of my former residents are currently competent professionals and I am happy every time they ask for my opinion on a case.

The health system depends on how we train residents both professionally and emotionally with an emphasis on humanitarian qualities, empathy, which are so vital in this profession.

Analyzing objectively and critically the didactic activity, I am aware that I have strengths but also weaknesses. My strength is the passion with which I teach medical semiology which I consider the queen of medical education and without which no medical graduate will be able to practice competently.

From the first months of didactic career I was actively involved in the elaboration of materials for semiology internships, clinical cases. Over the years I have collaborated in editing textbooks - revised editions of *Medical Semiology*, under the coordination of Prof. Carol Stanciu, *Course Notes*, fourth edition and *Sylabus. Essentials in gastroenterology* under the coordination of Prof. Anca Trifan. I am also a co-author of the medical treatise *Notions of Gastroenterology and Hepatology*, published by the Romanian Society of Gastroenterology and Hepatology, intended for residents and gastroenterologists.

I was a Guest Lecturer at numerous Workshops, Summer Schools and National/International Congresses organized either by our University or by the Romanian Society of Gastroenterology and Hepatology and other National Scientific Societies.

I am a member of the board of the Romanian Society of Gastroenterology and Hepatology, and member of the Society of Physicians and Naturalists Iași.

I have positively and promptly responded to the challenges launched by the Faculty of Medicine and participated in committees of residency, license, admission exam, teaching position promotion, specialty certificate examination, doctoral admission or tutorial commission.

3. SCIENTIFIC / RESEARCH ACTIVITY

I have been involved and trained in scientific and research activity since the first years of academic activity. My mentors showed me that competitiveness, interaction, collegiality and professionalism are the mandatory conditions for integration in a research team. All my scientific and research achievements are of an exceptional team guided by Prof. Carol Stanciu and Anca Trifan.

My doctoral thesis (MECTS No 3650/10.04.2009) has the topic *Iron disorders in chronic liver diseases* and it was written under the guidance of Prof. Carol Stanciu. The monography *Liver genetic diseases*. Ed. AXIS Academic Foundation, Iaşi, 2008, is the synthesis of the documentation and knowledge accumulated during the elaboration of the doctoral thesis.

The results of clinical and experimental research were used in articles published in journals and provided the **first data** on the incidence of HFE mutations in the population of North-Eastern Romania (Voicu PM, **Cojocariu Camelia**, et al. *Blood Cells Mol Dis.* 2009 Jan-Feb;42(1):14-5).

In 2005 we presented for the **first time** (at the National Congress of Gastroenterology and Hepatology) the results of sandostatin administration in variceal bleeding. Shortly after, we implemented in Romania the standardized treatment of acute variceal bleeding in cirrhotic patients according to international guidelines.

In 2011 we have published the **first case of CDI in Romania** in a 72-year old man with diarrhea, hypoproteinemia, edema and ascites (*J Gastrointestin Liver Dis* 2011) and I would like to emphasize our contribution to raising awareness about CDI. This was proven by numerous publications and citations but especially by the clinical implications regarding the approach of a patient with acute diarrheal disease.

I am the author/co-author of multiple papers published in ISI indexed journals such as (most relevant): *Expert Opin Pharmacother, Endoscopy, World Journal of Gastroenterology, Eur J Clin Microbiol Infect Dis, J Gastrointestin Liver Dis, Can J Gastroenterol Hepatol, World J Clin Cases, Medicina, Healthcare,* etc.

I would like to point out that all our articles are published in specialized indexed journals of medicine.

Recognition of the value of scientific and publishing activities is proven by the list of papers - ISI Thomson Reuters/BDI, number of citations, Hirsch index, books/chapters, oral and posters presentations at national and international scientific events.

- 57 book chapters (author/co-author), 1 monography
- Hirsch index – 11
- 342 citations in the ISI Thomson Reuters system
- 44 in extenso articles (15 first author) ISI journals, in Thompson Reuters indexed scientific journals
- 92 in extenso articles (24 first author) in journals indexed BDI and recognized by CNCSIS

- more than 150 papers accepted as poster/oral communication at national or international scientific events
- 28 guest presentations in the plenary of some national scientific events.

The scientific merits of our research team have been broadly and continuously recognized, as proven by the following international awards over times:

Awards of international scientific societies

AASLD Poster of distinction	Chiriac Ș, Stanciu C, Sîngeap AM, Sfarti C, **Cojocariu C**, Gîrleanu I, Cuciureanu T, Stoica O, Huiban L, Muzica CM, Livadariu RM, Trifan A. Relative adrenal insufficiency in the setting of acute on chronic liver failure - an organ dysfunction that could indicate futility. Digestive Disease Week, 2-5 iunie 2018, Washington
Italian Society for the Study of Ecstasy and Thrombosis - *Young Researcher Award.*	I. Girleanu, A. Trifan, **C. Cojocariu,** M. Dimache, A. Singeap, O. Stoica, C. Stanciu. Natural course of de novo partial portal vein thrombosis in patients with cirrhosis 5th International Congress on Coagulopathy in Liver Disease, Padua, 27-28 September 2013
United European Gastroenterology - *Oral Free Paper Prize*.	Irina Girleanu, Anca Trifan, Oana Cristina Stoica, **Camelia Cojocariu**, Ana Maria Singeap, Catalin Sfarti, Cezar Baluta, Carol Stanciu. Terlipressin-induced hyponatremia in cirrhotic patients with variceal bleeding. United European Gastroenterology Week, October 25-28, 2015, Barcelona
United European Gastroenterology - *Poster of excellence*.	Irina Girleanu, Anca Trifan, **Camelia Cojocariu**, Ana Maria Singeap, Ancuta Rohozneanu, Stefan Chiriac, Carol Stanciu. The effect of non-selective beta-blockers on survival in decompensated liver cirrhosis patients. United Eur Gastroenterol J. 2015; 3 (5S): A337. Week, October 25-28, 2015, Barcelona

The main research topics (the same ones from the clinical activity), which are actually presented in my thesis, are chronic liver disease (which we approached starting from etiology, evolution, complications and treatment methods), variceal and non-variceal gastrointestinal bleeding, *Clostridium difficile* infection.

I was actively involved in UMF" Gr. T. Popa" Iași **research grants** obtained through direct competition:

1. Prevalence of hereditary hemochromatosis and iron overload syndromes in the hospitalized population. (CNCSIS research grant, 33,541/2003); 2003–2006. Project director Prof. Carol Stanciu

2. Optimization of the detection protocol for families with hereditary non-polyposis colorectal cancer (HNPCC), in order to early diagnosis of premalignant lesions in asymptomatic subjects. (Romanian Academy research grant 169/2007); 2007-2008 (RON 6,000) 5. Interdisciplinary Platform in Molecular Medicine. (CNCSIS research grant, 40.110 / 2006); 2007-2009. Project director Prof. Carol Stanciu.

The clinical research has also resulted in participation in more than 20 multicenter, international clinical trials of phase II or III (especially chronic liver disease and inflammatory bowel disease), in which I was involved on the basis of a contract obtained through feasibility competition as co-investigator or study coordinator. My active involvement in these research projects has been extremely beneficial for the development of research skills, and the establishment of individual goals, a model for developing a project, and a clinical protocol.

From the first years I was actively involved in the editorial activity: I was deputy editor-in-chief of the Journal for Continuing Medical Education –a scientific journal recognized by CNCSIS (The experience gained in this editorial activity was extremely important for the development of my skills of documentation and evaluation of an article, improvement of the elaboration of a scientific paper). I was also deputy editor of the newsletter of the Romanian Society of Gastroenterology and Hepatology - Info SRGH, editor of the treatise edited by C Stanciu, A Trifan, I Sporea. *Inflammatory bowel diseases*, Ed "Gr T Popa", Iasi, 2014. Moreover, I have contributed substantially to the technical drafting of all books published by the team of the department of medical semiology and gastroenterology and for 5 years I was part of the editorial staff of the Medical-Surgical Journal of the Society of Naturalists of Iasi.

The recognition of my scientific activity is also demonstrated by the quality of member of the Editorial Board for the Artificial Intelligence in Medical Imaging, **peer-reviewer** for international indexed journals (*Journal of Clinical and Translational Hepatology*, *Asian Journal of Research and Reports in Gastroenterology*, *World Journal of Hepatology*, *International Medical Case Reports Journal*, *Journal of Pharmaceutical Research International*, *Frontiers in Medicine*) as well as by the numerous prizes won at national/international events of the professional societies or UEFISCDI (selection).

My research and journalistic activity was and is closely related to my clinical activity and is oriented towards the main pathologies - gastroenterology, hepatology - with which they come in contact in the clinical activity. The papers published in specialized journals and in the volumes of scientific publications prove the capacity for synthesis as well as the medical and scientific level acquired.

I want to build an academic career and an excellent professional reputation which will ensure success and increased visibility to the Department of Medical Semiology and Gastroenterology and in this way to the Faculty of Medicine “Grigore T. Popa” Iași.

The opportunity of selecting the candidates and coordinating the PhD thesis would greatly motivate me to continue my development as an academic professor.

Last but not least, one of the most important duty for me will be to proudly promote the our University. I consider that establishing new contacts and improving the existing ones are the best ways, for me and my PhD students, to get involved in national and international projects, to access funds, develop and obtain recognition.

Chapter 1.

MAIN CAUSES OF CHRONIC LIVER DISEASES –TWO-WAY TRAFFIC TO LIVER CIRRHOSIS

1.1. INTRODUCTION

Regardless of its etiology chronic hepatitis represents a major global health problem due to its high prevalence and especially because of its progression to advanced liver disease and liver cirrhosis whose natural history is fraught with numerous complications and poor prognosis. The causes of chronic liver disease (CLD) are numerous but the main causes in the developed countries are represented by alcoholic liver disease, chronic hepatitis B and C virus infection, and non-alcoholic fatty liver disease (NAFLD) (Asrani et al., 2019). However, in addition to these major and well-known causes there are other rarer etiologies with a major impact on CLD epidemiology. For instance, hereditary hemochromatosis (HHC) is generally considered a rare disease but taking into account the geographical distribution of the cases it is a quite common cause of chronic liver injury in the Caucasian population (Heidelbaugh and Bruderly, 2006).

How and to what extent can we influence the causes and evolution of chronic liver disease?

Given the high morbidity and mortality rates associated with CLD worldwide there is a great need for action to reduce the number of cases and to slow the progression to liver cirrhosis and its complications. Therefore, prophylactic strategies seem to be the most appropriate method for decreasing CLD morbidity and mortality. The etiological treatment is the turning point that can stop or slow down the evolution of the chronic disease. Thus, the acknowledgment of the common causes of CLD and the possible therapeutical measures will lead to a beneficial impact on the evolution of most cases.

Alcoholic liver disease (ALD**)**. Alcohol consumption is one of the main causes of chronic liver injury and is accountable for an estimated 27% of liver-related deaths worldwide (Mokdad et al., 2016). Most deaths attributed to alcohol consumption are secondary to cardiovascular disease, trauma, gastrointestinal disease - especially liver cirrhosis, and cancer. Epidemiological studies showed that in Europe, 41% of deaths from liver disease are certainly attributed to alcohol, and another 46% of unspecified etiology could be also related to alcohol abuse (Sheron, 2016). The prevention and treatment of ALD are extremely difficult as long as alcohol consumption is increasing. Moreover, there has been a significant increase in global alcohol consumption during the pandemic and unfortunately it is likely to remain at least constant (Jovan et al., 2021). Several strategies can help communities to create social and physical environments that discourage heavy alcohol consumption such as psychological counseling programs, regulation of alcohol outlet density, increasing alcohol taxes, and enhanced enforcement of laws prohibiting sales to minors. These measures should theoretically reduce alcohol-related fatalities, costs, and other harms.

Chronic hepatitis B (HBV) and C (HCV) virus infection. According to the data provided by the World Health Organization, there are 4 million new cases of acute HBV hepatitis detected annually and a total of 75 million individuals are diagnosed with HCV infection (WHO. Global hepatitis report, 2017). However, a downward trend of the prevalence of chronic HBV and HCV virus infection is expected in the coming years, as a consequence of the implementation of HBV vaccination programs, the effectiveness of screening, and increasing access to antiviral therapy for HCV.

The implementation of HBV vaccination in countries with high endemicity is the most effective measure that has led to a dramatic decrease in new cases (Sun et al., 2013). The use of nucleoside analogs, strict control of transfusible products and prevention of risky sexual behavior have also significantly contributed to lowering the prevalence of HBV infection.

Antiviral therapy for chronic HCV infection registered a dramatic evolution in the last years, leading to an extraordinary improvement of the rate of sustained virological response. Since the revolutionary discovery of the new all-oral direct-acting antivirals, the rate of obtaining the sustained virological response (SVR) increased from about 50% using interferon -based regimens to over 95%.

Nonalcoholic fatty liver disease (NAFLD) has become pandemic in the last decade with rising morbidity and mortality worldwide. Its associated complications and extrahepatic manifestations contribute to a high burden, both for patients and healthcare systems. Given the rapidly changing epidemiology of the etiology of chronic liver disease following increased access to curative treatment for chronic hepatitis B and C, NAFLD is beginning to become a major concern for hepatologists everywhere. Even though there is currently no effective treatment for NAFLD the extrahepatic manifestations of this disease can be treated if recognized early thus reducing the mortality and morbidity of NAFLD. Still, as long as there is no effective treatment proven to control the disease it is unlikely to expect a significant reduction in incidence rates in the coming years (Raza et al., 2021).

Hereditary hemochromatosis is a common cause of chronic liver disease in the Caucasian population (Heidelbaugh and Bruderly, 2006). Early diagnosis and initiation of treatment before the onset of irreversible organic lesions can influence the progression of the disease to liver cirrhosis but does not eliminate the risk of hepatocellular carcinoma (HCC). Data from the literature describes the risk of HCC over 200 times higher in persons with HHC than in the general population.

Liver fibrosis (LF). The evaluation of LF is the most important criteria for the grading of CLD and the assessment of the rate of progression to liver cirrhosis. Hepatic fibrosis is a dynamic complex that causes the growth of extracellular matrix components, the activation of extracellular matrix-producing cells, the release of cytokines, and tissue remodeling. Slowing or even regression of LF is an important condition for improving the prognosis of patients with CLD. LF has long been considered a one-way, linear process. There is growing evidence that the evolution of fibrosis is bidirectional and regression of fibrosis has been documented in patients treated with phlebotomies for hemochromatosis, and more recently after successful suppression and eradication of chronic hepatitis B and C infections (Carrat et al., 2019).

In light of the above, in this chapter I will present our results regarding the common causes of CLD and I will summarize the scientific activity related to the non-invasive methods for evaluating liver fibrosis in patients with CLD.

The scientific expertise in this field is supported by the scientific and editorial activity:

	Articles published in ISI indexed journals	**WOS citations**
1.	Carol Stanciu, Cristina Maria Muzica, Irina Girleanu, **Camelia Cojocariu**, Catalin Sfarti, Ana Maria Singeap, Laura Huiban, Stefan Chiriac, Tudor Cuciureanu, Anca Trifan. An update on direct antiviral agents for the treatment of hepatitis C. *Expert Opin Pharmacother* 2021; 22(13): 1729-1741. **IF – 3.88**	5
2.	Laura Huiban, Carol Stanciu, Cristina Muzica, Tudor Cuciureanu, Stefan Chiriac, Sebastian Zenovia, Vladut Mirel Burduloi, Oana Petrea, Ana Maria Singeap, Irina Girleanu, Catalin Sfarti, **Camelia Cojocariu**, Anca Trifan. Hepatitis C virus prevalence and risk factors in a village in Northeastern Romania- A Population-based screening-The first step to viral micro-elimination. *Healthcare* 2021; 9(6): 651. **IF – 1.58**	

	Articles published in ISI indexed journals	WOS citations
3.	Gheorghe L, Iacob S, Csiki IE, Huiban L, Cojocaru M, **Cojocariu C**, Nemteanu R, Girleanu I, Sirli R, Singeap AM, Pop C, Dumitrascu DL, Vadan R, Iacob R, Diculescu M, Trifan A, Sporea I, Gheorghe C. The Prevalence of HCV Infection and Risk Factors in a Hospital-Based Population Screening, a First Step to the Micro-Elimination of HCV Infection in Medical Institutions from Romania - Results of the HepC ALERT Study. *J Gastrointestin Liver Dis*. 2020 Dec 12;29(4):587-593. **IF – 0.641**	2
4.	**Cojocariu C**, Singeap AM, Girleanu I, Chiriac S, Muzica CM, Sfarti CV, Cuciureanu T, Huiban L, Stanciu C, Trifan A. Nonalcoholic Fatty Liver Disease-Related Chronic Kidney Disease. *Can J Gastroenterol Hepatol.* 2020 Dec 29;2020:6630296. **IF - 2.9**	1
5.	Trifan A, Rotaru A, Stafie R, Stratina E, Zenovia S, Nastasa R, Huiban L, Cuciureanu T, Muzica C, Chiriac S, Gîrleanu I, Sîngeap A-M, Sfarti C, **Cojocariu C**, Stanciu C. Clinical and Laboratory Characteristics of Normal Weight and Obese Individuals with Non-Alcoholic Fatty Liver Disease. *Diagnostics.* 2022; 12(4):801. **IF- 3.706**	
6.	Nastasa R, Stanciu C, Zenovia S, Singeap AM, Cojocariu C, Sfarti C, Girleanu I, Chiriac S, Cuciureanu T, Huiban L, Muzica CM, Trifan A. The Prevalence of Liver Steatosis and Fibrosis Assessed by Vibration-Controlled Transient Elastography and Controlled Attenuation Parameter in Apparently Healthy Romanian Medical Students. *Diagnostics* (Basel). 2021 Dec 13;11(12):2341. **IF – 3.24**	
7.	Tudor Cuciureanu, Carol Stanciu, Sebastian Zenovia, Cristina M. Muzica, Robert Nastasa, Laura Huiban, **Camelia Cojocariu,** Ana-Maria Singeap, Irina Girleanu, Stefan Chiriac, Catalin Sfarti, Vladut Mirel Burduloi, Anca Trifan. Non-alcoholic fatty liver disease and extra-hepatic malignancies. *JBUON* 2021; 26(6): 2421-2424. **IF – 2.53**	
8.	Stefan Chiriac, Carol Stanciu, Irina Girleanu, **Camelia Cojocariu**, Catalin Sfarti, Ana-Maria Singeap, Tudor Cuciureanu, Laura Huiban, Cristina Maria Muzica, Sebastian Zenovia, Robert Nastasa, Anca Trifan. Nonalcoholic Fatty Liver Disease and Cardiovascular Diseases: The Heart of the Matter. *Can J Gastroenterol Hepatol.* Volume 2021, Article ID 6696857, **IF - 2.9**	7
9.	Sebastian Zenovia, Carol Stanciu, Catalin Sfarti, Ana Maria Singeap, **Camelia Cojocariu**, Irina Girleanu, Mihaela Dimache, Stefan Chiriac, Cristina Maria Muzica, Robert Nastasa, Laura Huiban, Tudor Cuciureanu, Anca Trifan. Vibration-Controlled Transient elastography and Controlled Attenuation Parameter for the diagnosis of liver steatosis and fibrosis in patients with nonalcoholic fatty liver disease. *Diagnostics* (Basel) 2021; 11(5): 787 **IF – 3.24**	1
10.	Ana-Maria Sîngeap, Carol Stanciu, Laura Huiban, Cristina Maria Muzica, Tudor Cuciureanu, Irina Girleanu, Stefan Chiriac, Sebastian Zenovia, Robert Nastasa, Catalin Sfarti, **Camelia Cojocariu**, Anca Trifan. Association between Nonalcoholic Fatty Liver Disease and Endocrinopathies: Clinical Implications. Can J Gastroenterol Hepatol. Volume 2021, Article ID 6678142, **IF - 2.9**	6
11.	Muzica CM, Sfarti C, Trifan A, Zenovia S, Cuciureanu T, Nastasa R, Huiban L, **Cojocariu C**, Singeap AM, Girleanu I, Chiriac S, Stanciu C. Nonalcoholic Fatty Liver Disease and Type 2 Diabetes Mellitus: A Bidirectional Relationship. *Can J Gastroenterol Hepatol* 2020 Dec 28;2020:6638306. **IF - 2.9**	7
12.	Mihai F, Trifan A, Stanciu C, Singeap AM, Cucuteanu B, Lupascu Ursulescu C, Pop C, Girleanu I, Cuciureanu T, Negru D, **Cojocariu C**. Liver Remodeling on CT Examination in Patients with HCV Compensated Cirrhosis Who Achieved sustained virological response after Direct-Acting Antivirals Treatment. *Medicina* (Kaunas). 2020 Apr 10;56(4):171. **IF- 2.34**	1
13.	Trifan A, Stratina E, Rotaru A, Stafie R, Zenovia S, Nastasa R, Huiban L, Sfarti C, **Cojocariu C**, Cuciureanu T, Muzica C, Chiriac S, Girleanu I, Singeap A-M, Stanciu C. Changes in Liver Steatosis Using Controlled Attenuation Parameter among Patients with Chronic Hepatitis C Infection Treated with Direct-Acting Antivirals Therapy Who Achieved Sustained Virological Response. *Diagnostics*. 2022; 12(3):702. **IF- 3.706**	
14.	Trifan A, **Cojocariu C,** Stanciu C. Liver biopsy or transient elastography? First, not harm! *J Gastrointestin Liver Dis*. 2012 Sep;21(3):325-6. **IF 1.71**	1
15.	Trifan A, Sfarti C, **Cojocariu C**, Dimache M, Cretu M, Hutanasu C, Stanciu C. Increased liver stiffness in extrahepatic cholestasis caused by choledocholithiasis. *Hepat Mon.* 2011 May 1;11(5):372-5. **IF 2.35**	20

Articles published *in extenso* in BDI indexed journals		
	Camelia Cojocariu, Carol Stanciu, Anca Trifan. Evaluation and analysis of the trend of incidence and prevalence of non-alcoholic fatty liver disease. *Ro Med J*. 2020;67(Suppl) DOI: 10.37897/RMJ.2020. S. 2	
	Anca Trifan, Sebastian Zenovia, Cristina Muzica, Laura Huiban, Catalin Sfarti, **Camelia Cojocariu**, Ana Maria Singeap, Irina Girleanu, Ermina Stratina, Adrian Rotaru, Robert Nastasa, Carol Stanciu. Short-term changes of liver fibrosis in patients with HCV genotype 1b-related compensated cirrhosis after sustained virologic response. *Rev Med Chir* 2021;125(3):365-375	
	Anca Trifan, **Camelia Cojocariu,** C. Sfarti, Ana-Maria Sîngeap, C. Stanciu. Non-invasive evaluation of liver fibrosis in chronic hepatitis C. *Rev Med Chir* 2012;116(1):135	4
	Pia-Manuela Voicu, **Camelia Cojocariu**, Elena Petrescu-Dănilă, C. Stanciu, M. Covic, M. Rusu, Anca Trifan: Hereditary Hemochromatosis in North-Eastern Romania. *Rev Med Chir* 2010; 114(4):982-987	
Book chapters		
	Camelia Cojocariu, Anca Trifan, Sfarti C, Stanciu C. Markeri non invazivi serologici pentru evaluarea fibrozei hepatice. În: Stanciu C. *Actualităţi în gastroenterologie şi hepatologie*, Ed Junimea, Iaşi, 2010: 11-20.	
	Florin Mihai, Anca Trifan, Ana Maria Sîngeap, **Camelia Cojocariu**, Dragoș Negru. Utilizarea tomografiei computerizate în evaluarea pacienților cu ciroză hepatică. În *Noi concepte în gastroenterologie și hepatologie – actualități*, ISBN: 978-606-544-430-0 Ed Gr T Popa, Iași 2016: 343-356	
	Camelia Cojocariu, Gabriela Ștefănescu, Anca Trifan. *Hemocromatoza ereditară* În Gastroenterologie și Hepatologie clinică, sub red. Anca Trifan, C Gheorghe, D Dumitrascu, et al, Editura medicală 2018: 596-605; ISBN 978-973-39-0846-3	
	Over 8 chapters in *News in the diagnosis and treatment of chronic viral hepatitis*. "Iuliu Haţieganu" University Medical Ed, Cluj Napoca, published between 2009-2012	
Total WOS citations		**55**
Cumulative impact factor/direction		**40.523**

1.2. HEREDITARY HEMOCHROMATOSIS – UNDERDIAGNOSED CONDITION

1.2.1. Introduction

HHC is an iron overload disorder and is probably the most common inherited disorder of people of northern European ancestry. It was thought to be a very rare condition for a long time, only occurring in 1 in 20 000 persons (Finch, and Finch, 1955).

In 1996, the hereditary hemochromatosis gene (HFE) was identified (located on chromosome 6) and it was a major breakthrough in the understanding of HHC (Feder et al, 1996). At that time, two mutations were identified in the HFE gene - C282Y and H63D, and the pathogenesis of the disease appeared to be fully elucidated. At the beginning of the new millennium, it was unanimously accepted that HHC is the most common metabolic disease in northern Europe, with a frequency of 1 case in 200-300 people, and C282Y homozygous status is the defining genotype of the disease. Data from epidemiological studies estimate that more than 50% of people with a genetic predisposition will develop some form of the disease sometime (Bacon, 2001). However, not all patients with congenital HHC phenotype have mutations in this gene, especially in southern European countries, and many patients with HHC genotype do not have iron overload syndrome. Certainly, the mutation is necessary but not sufficient for the disease expression and the homozygous status means genetic predisposition and not the disease itself. All these findings required the use of a new term -

non-HFE hemochromatosis - used for iron overload cases with no mutations in the HFE gene (Beutler, 2003; Pietrangelo, 2004).

Advances in genetics have modified the diagnostic algorithm of HHC, demonstrating that the phenotypic expressions of a mutation in genes involved in iron metabolism are variable; thus OMIM (Online Mendelian Inheritance in Man) states that there are four forms of HHC, each of them caused by mutations in another gene (Pietrangelo, 2004).

The characteristics of classical HHC - which remains the most common form in the European population, are also found in other phenotypic forms. Type 2A, 2B, and 3 have the same pathogenic mechanism, clinical picture, and autosomal recessive transmission as HHC, expressed as forms of the same syndrome (Harrison, and Bacon, 2003; Pietrangelo, 2003; Pietrangelo, 2004). Type 2 (juvenile hemochromatosis) is associated with mutations within the genes encoding hemojuvelin or hepcidin (Pietrangelo, 2004), while type 3 is associated with mutations within the genes encoding transferrin receptor 2 (Pietrangelo, 2003). Type 4 (ferroportin disease) has different epidemiology, natural history, and molecular pathogenesis from HHC, so probably a different diagnostic and screening approach; it is caused by mutations in the SLC40A1 gene (originally called SLC11A3) (Pietrangelo, 2004).

Careful interpretation of an iron overload syndrome, early diagnosis of HHC before the onset of irreversible organic lesions, and family screening are prerequisites for the prognosis of patients with this condition, which is still underdiagnosed worldwide.

Iron parameters changes in chronic liver disease and iron-overload syndromes are my first major research topic, and most of published articles where during my doctoral studies; we have published **the first data regarding the prevalence of HFE mutations in Romania.**

1.2.2. Hereditary hemochromatosis in North-Eastern Romania

Background & aim. Hereditary hemochromatosis is the most frequent genetic disease in some regions of Europe such as Scandinavia, Ireland, Great Britain, and France, where the disease is a public health problem. We aimed two objectives in our study: 1) to estimate the expectancy of the disease by genotyping a sample of the normal population for the most frequent HFE mutations, C282Y and H63D, and 2) to use and optimize methods of discovering the disease among patients.

Materials and methods. Population samples: For the prevalence of the C282Y and H63D alleles in the healthy population the sample consisted of 200 individuals. Forty were healthy blood donors and 160 were ambulatory and hospitalized patients with diagnoses not suggesting hemochromatosis, liver diseases, diabetes, or rheumatism. We included these patients in the sample to vary their geographical origin. Eighty -five originated from the Iasi district (including the blood donors), 33, 19, 23, 24 and 16 individuals originated from the districts of Vaslui, Bacau, Botosani, Suceava, and Neamt respectively. 105 were males and 95 were females. All the subjects were genotyped for C282Y and 146 subjects were genotyped for H63D.

For the screening of the disease, the sample consisted of 549 patients hospitalized in the Institute of Gastroenterology and Hepatology Iasi, diagnosed with chronic hepatitis or cirrhosis. 208 were men and 241 were women. The age of subjects is shown in Figure 1.1.

The patients were tested for plasma iron level, transferrin saturation index, and ferritin level in addition to other investigations (including liver biopsies in some cases) specific to their pathology. Ten patients with high cumulated blood iron levels, transferrin saturation index, and ferritin or a hepatic iron overload, discovered at the liver biopsy, were genotyped for C282Y and H63D. One of them was sequenced for mutations in ferroportin, hepcidine, and hemojuveline genes.

Informed consent was obtained from all the subjects in the two groups mentioned.

Biochemical investigations: Iron level and the total capacity of iron-binding were

determined colorimetrically by using commercial kits. Transferrin saturation index was calculated from the two values aforementioned. Ferritin was determined by using an immunochemical method with a commercial kit.

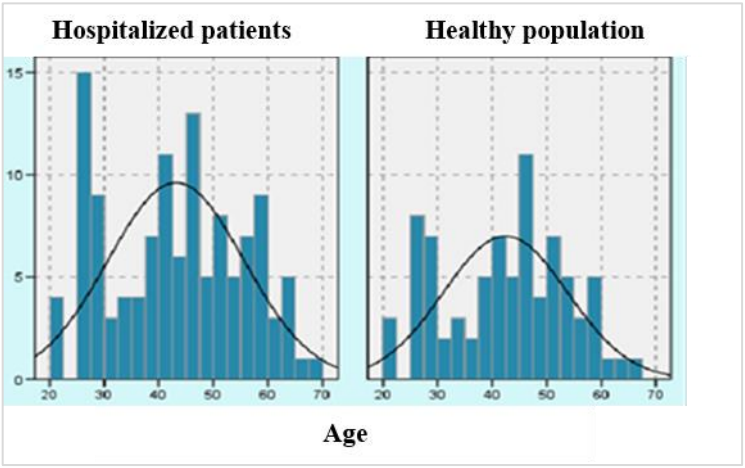

Fig. 1.1. Age distribution in healthy population and hospitalized patients

Genotyping: The C282Y and H63D mutations were tested by a PCR- RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis as described (Jouanolle et al., 1996; Voicu et al., 2009).

Parts of the HFE gene were PCR (polymerase chain reaction) amplified using appropriate primers and the amplicon obtained was digested with restriction enzymes: RsaI for C282Y and MboI for H63D. The fragments obtained were analyzed for their sizes after electrophoretic separation in agarose gels. The sequencing of ferroportin, hepcidin, and hemojuvelin genes was carried out in the molecular biology laboratory of the Lille University Hospital (France) by one of us. The genomic DNA was extracted with a Qiagen kit, The entire coding regions and splice sites of all these genes were sequenced using a terminator cycle sequencing reaction kit and a 3130 XL Genetic Analyzer (Applied Biosystems).

Results. Our first goal was to evaluate how frequently we expect a hemochromatosis case in our population. A population sample of 200 persons from the Moldavian territory was organized, consisting of individuals not suspected of hemochromatosis. The distribution of HFE mutant alleles in our population group designed to evaluate the prevalence of mutations in the HFE gene are presented in Table 1.I.

Table 1.I. Mutant genotypes of HFE and their frequency in Moldavia

Genotype	Number of subjects tested	Number of subjects with genotype	Percentage
C282Y/+	200	7	3.5
C282Y/C282Y	200	0	0
H63D/+	140	28	20
H63D/H63D	140	4	2.9
C282Y/H63D	140	0	0

The allele frequency (nr. of chromosomes carrying the mutant allele x 100/total nr of chromosomes) calculated from our data (Table 1.I) are 1.75 (95% confidence interval: 0.7 - 3.7) for the C282Y mutant allele and 13.25 (95% confidence interval 10.4 - 16.5) for the H63D allele. Also, we found an expectancy of one homozygous (possibly ill) C282Y/C282Y person out of 816 persons. This would be so if the penetrance of the mutant allele was 1. However, penetrance is lower than 1 and varies in different populations. As we do not know yet penetrance (number of persons with phenotype/number of persons with genotype) in our

population, we can only assess that we expect at most one person suffering from hemochromatosis among 816 persons. As to the compound heterozygote C282Y/H63D, its expectancy is much higher (one out of 100 individuals), but this genotype very rarely causes hemochromatosis (generally a mild iron overload with few clinical consequences).

Our second goal was to apply and optimize a selection method to discover among clinically suspected patients, those who suffered from hemochromatosis. As genotyping is more expensive and does not always discover the disease, we tested 549 patients, whose clinical status suggested possible hemochromatosis for iron-overload parameters generally recommended: plasma iron level, transferrin saturation index and ferritine level. We selected eventually 10 patients cumulating iron blood levels higher than 160 mg/dL, transferrin saturation index higher than 70 and a ferritin level higher than 500 ng/mL for genotyping. HFE genotype in our subjects is shown in Figure 1.2.

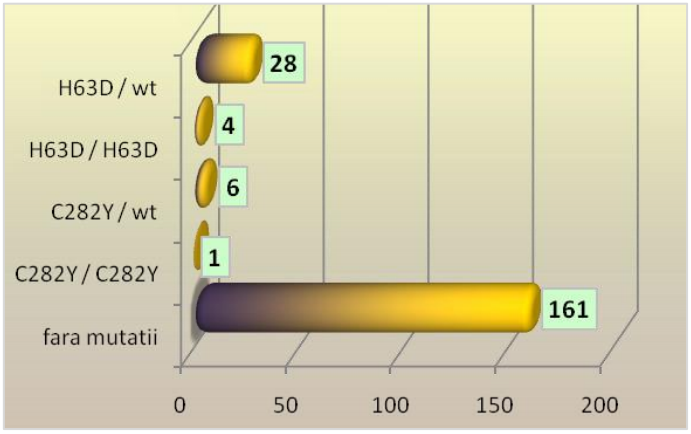

Fig. 1.2. HFE genotype in studied patients

Three of subjects were found to carry mutations: one was a HFE C282Y/C282Y homozygote (Figure 1.3), another one was a HFE C282Y/H63D compound heterozygote and the third one had no mutations in HFE. After sequencing other genes (ferroportin, hepcidin and hemojuvelin genes), a homozygous G320V mutation was finally found in the hemojuvelin gene.

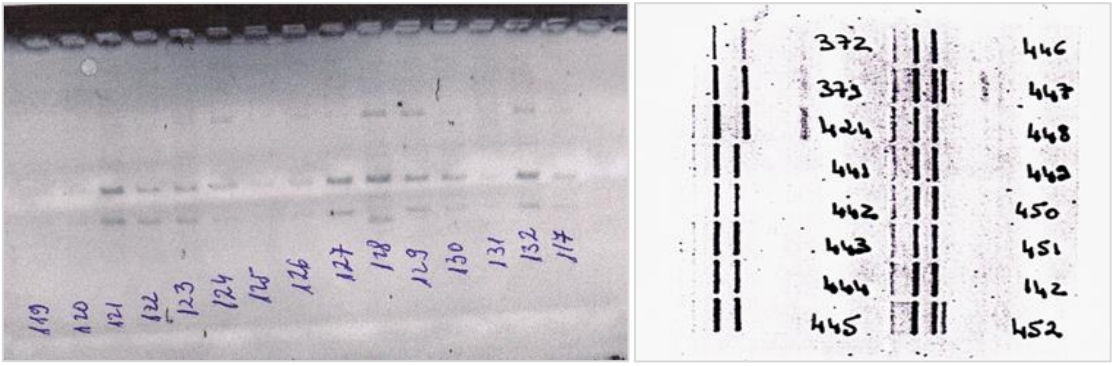

Fig. 1.3. RsaI restriction enzymes for C282Y – homozygote C282Y/C282Y

Discussion. In this study, the objective was to diagnose hemochromatosis among our patients. Firstly, we aimed to estimate the status of the most frequent mutations causing hereditary hemochromatosis, the HFE C282Y and H63D mutant alleles, in the broad population of our region. We established a population sample of 200 people from most part of the districts of the region. Subjects composing the sample were healthy blood donors and (in majority) patients with various diseases, but not the ones possibly suggesting hemochromatosis. It did not

cause a bias of the data since the mutations we found were in heterozygous condition, known as not having a phenotype. The allelic frequency of the HFE- C282Y found in our population, 1.75%, can be compared with allelic frequencies in other regions in Eastern Europe: northwestern Poland 4.73% (Raszeja-Wyszomirska et al., 2008), southern Poland 3.11 % (Moczulski et al., 2001), Moscow region 3.4 % (Potechina et al., 2005), Czech Republic 3.45 % (Cimburova et al., 2005), Hungary 3.40 % (Andrikovics et al., 2001), Serbia-Montenegro 1.60 % (Saric et al., 2006), Bosnia-Herzegovina 2.25 % (Terzic et al., 2006) Bulgaria (Sofia Region) 0 % (Ivanova et al., 1999), Greece 0.3 % (Papazoglu et al., 2003).

Our data fits the interesting, but not very well understood pattern of northwest-to-southeast decline of the allelic frequency available in Europe. This may account for the population migrations in the past. The allelic frequency of H63D in our population (13.25) is close to those found in almost all European and non-European populations. Our data shows a maximum expectancy of one individual affected by severe hemochromatosis in approximately 800 people, which makes hemochromatosis still a rare disease, but the most frequent genetic disease in our region and justifies special attention from healthcare services.

We also tested a practical method to discover HHC in patients with clinically suspected illness. We applied the established rules as to discover the phenotype and we found 10 patients having a hemochromatosis phenotype in a sample of 549 patients. They were genotyped for the HFE mutations and one of them was a C282Y/C282Y homozygote. This was close to expectancy in the general population, although we expected more in a group of pathology selected patients. This could be due to the lower than 1 penetrance of the mutant allele as well as to flaws in the phenotype or genotype determination, resulting in missing of some cases.

A similar evaluation in a group of patients from southwestern Romania, four C282Y homozygotes were found among 21 cases of iron overload (Neghina et al., 2009). As to the other 7 cases of phenotypic hemochromatosis in our group, some may have been secondary hemochromatosis (one of them had a myelodysplastic syndrome) and others may have had mutations in other genes. One of them proved this by sequencing other genes and finding a homozygous G320V mutation in the hemojuvelin gene, the second case to carry this mutation in Romania, after another one recently published (Militaru et al., 2010). We have not yet discovered the cause of iron overload in the remaining 5 patients.

Conclusions. Allelic frequency of the HFE mutant allele C282Y in Moldavia is about 1.75% and the expectancy of a severe form of hemochromatosis is one in about 800 people. Three cases of hereditary hemochromatosis were found by looking for its phenotype among 549 patients: one was a HFE-C282Y homozygote, a second was a HFEC282Y/H63D compound heterozygote and a third was a HJV-G320V homozygote.

It is generally considered that HHC in Romania is a rare disease, with variable incidence compared to other European countries. Our data suggest that HHC is still underdiagnosed, which is why we believe that any biological change in iron parameters must be carefully analyzed.

1.3. CHRONIC HEPATITIS C INFECTION – THE SILENT EPIDEMIC

1.3.1. Introduction

Chronic HCV infection is one of the main causes of chronic liver disease, a public health problem and a socio-economic burden worldwide. There are almost 71 million persons with positive HCV worldwide. The mean prevalence in Europe is 0.65%, and, unfortunately, Romania is on the first place with 2.5% HCV prevalence, corresponding to 550,000 patients with positive viral loads (Han et al., 2019). The prevalence of anti-HCV antibodies in

populations from Central and Eastern Europe varies between 0.27 and 3.5%, the number of people infected with HCV in the general population being approximately 1.16 million (Madaliński et al., 2015).

October 2020 marked a milestone in hepatology, when the Nobel Prize in Medicine, an award that recognizes a quantum leap in virology, was awarded for the discovery of HCV. This discovery revolutionized the clinical care of millions of patients by aiding in the development of effective treatment strategies to cure this infection.

In 2013, sofosbuvir (SOF), a highly potent inhibitor of the HCV nonstructural protein 5B (NS5B) polymerase, was approved by the Food and Drug Administration (FDA) and was considered a genuine breakthrough in the course of developing efficient strategies for HCV therapy (Pawlotsky, 2020).

The development of direct-acting antiviral therapy (DAA) for chronic HCV infection is one of the greatest advances in clinical medicine in the past decade with numerous benefits. DAAs demonstrated a high efficacy with SVR rates above 95%, with minimal side effects, good tolerability, short duration of treatment (8–12 weeks) and oral drug administration. The excellent results of the introduction of the new DAAs in the eradication strategies in patients with chronic HCV infection, have led to the issuance of an ambitious WHO proposal to eliminate HCV as a public health threat by 2030 (World Health Organization, 2017).

1.3.2. The Prevalence of HCV Infection and Risk Factors in a Hospital-Based Population Screening, one Step to the Micro-Elimination of HCV Infection in Medical Institutions from Romania – Results of the HepC ALERT Study

Background & Aim. Micro-elimination, by targeting smaller and clearly delineated HCV risk groups, allows faster and better delivery of interventions. The development of possible micro-elimination scenarios breaking down national elimination goals into individual population segments enables policymakers to understand current disease landscapes on a hospital-based or regional level. We aimed to screen HCV in specific high-risk populations in certain sub-regions of Romania and link them to antiviral treatment. This integrated project of testing-diagnosis -treatment performed in over 20 medical institutions in Romania had as its objectives: micro-elimination of HCV at an institutional level among the patients that are admitted to medical units in Romania, prevention of advanced HCV liver disease, prevention of HCV transmission among the healthy population and updating the epidemiological data regarding HCV in Romania.

Methods. A multicenter prospective study (HepC ALERT, HepC Awareness & Test-Linkage to care–Epidemiological Research-Treatment) was conducted among hospitalized or ambulatory adult patients from March 2019 to March 2020 in more than 20 medical institutions from 4 Romanian cities (Bucharest, Iasi, Timisoara, Cluj-Napoca). Each center had included all consecutive patients addressed for routine monitoring or new patients for consultation, after signing the informed consent. A rapid diagnostic test for HCV diagnosis was performed to all admitted patients and the positive ones were sent to gastroenterology departments to confirm the active infection, staging and treatment prescription (linkage to-care). Demographic data on age, gender, area of residence, ethnicity, marital status, education, employment, and data on risk factors for HCV infection were collected through an epidemiologic questionnaire. All data were stored while performing the test in an anonymized database that could have been accessed online at any time.

Statistical analysis. The prevalence of HCV positive patients was calculated with a 95% confidence interval (CI). Qualitative or quantitative variables were analyzed using nonparametric tests, the Chi-square test, Kruskal-Wallis test or the Mann Whitney U test, as appropriate. Using logistic regression, odds ratio (OR) together with the corresponding 95%

CI were computed for the majority of investigated variables. All statistical tests were two-sided and a level of $p \leq 0.05$ was used to indicate statistical significance. Statistical analysis was performed using the Stata/SE 11 software.

Results. A total of 25,141 subjects signed the informed consent and were consequently enrolled into the study. The prevalence of anti-HCV in the population that presented to the medical institutions in four big cities from Romania was 1.39% (95% CI: 1.25-1.54). The distribution of patients, according to the 4 cities, was as follows: Bucharest (12,875 tested persons, 1.26% HCV prevalence); Cluj-Napoca (1,281 tested persons, 2.11% HCV prevalence); Iasi (6,896 tested persons, 1.38% HCV prevalence); Timisoara (4,089 tested persons, 1.59% HCV prevalence) (p=0.054).

The study population consisted of 15,802 females and 9,209 males. The HCV prevalence was similar among females and males (1.47% vs. 1.27%, p=0.199). The HCV prevalence for inhabitants of rural areas was 2.32% compared to 1.15% for subjects living in urban and metropolitan areas (p<0.001). The mean age of participants was 53.1±16.5 years. The prevalence of HCV infection increased markedly with age (p <0.001) and the prevalence of HCV positive antibodies in the study cohort according to the area of development is shown in Table 1.II.

Table 1.II. HCV prevalence in the screened subjects according to the area of development

N	Area of development	Tested subjects, N	HCV Prevalence	
			%	95% CI
1	North West (Bihor, Bistrița-Năsăud, Cluj, Maramureș, Satu-Mare, Sălaj)	1,124	1.96	1.29 – 2.96
2	West (Arad, Caraș-Severin, Hunedoara, Timiș)	3,887	1.44	1.11 – 1.87
3	South West – Oltenia (Dolj, Gorj, Mehedinți, Olt, Vâlcea)	636	1.57	0.85 – 2.90
4	South Muntenia (Argeș, Călărați, Dâmbovița, Giurgiu, Ialomița, Prahova, Teleorman)	1,855	2.26	1.68 – 3.05
5	South Est (Brăila, Buzău, Constanța, Galați, Tulcea, Vrancea)	1,697	1.59	1.09 – 2.31
6	North Est (Bacău, Botoșani, Iași, Neamț, Suceava, Vaslui)	7,022	1.41	1.16 – 1.71
7	Center (Alba, Brașov, Covasna, Harghita, Mureș, Sibiu)	178	0.56	0.08 – 3.90
8	Bucharest - Ilfov	8,742	1.05	0.86 – 1.29
	Total screening cohort	25,141	1.39	1.25 – 1.54
Test Chi^2 (comparison of positive tests between regions) – p = 0.003				

Table 1.III. shows the prevalence of HCV infection according to the risk factors identified by the addressed questionnaire.

Table 1.III. Prevalence of anti-HCV antibodies by different risk factors

Risk factors (answers from the questionnaire)	Tested patients, N	HCV Prevalence		p (Chi^2)
		%	95% CI	
Previous known KBV/HDV				
No	24,391	1.35	1.22 – 1.51	0.007
Yes	750	2.53	1.62 – 3.94	
Known HCV (+) family members				
No	23,983	1.28	1.15 – 1.43	< 0.001
Yes	1,158	3.63	2.69 – 4.87	
Known HBV / HDV (+) family members				
No	23,911	1.35	1.21 – 1.50	0.013
Yes	1,230	2.20	1.51 – 3.18	

Risk factors (answers from the questionnaire)	Tested	HCV Prevalence		p (Chi^2)
Decreased relatives with LC / HCC				
No	22,351	1.34	1.20 – 1.50	0.053
Yes	2,790	1.79	1.36 – 2.36	
Professional exposure to blood products				
No	20,413	1.63	1.46 – 1.81	< 0.001
Yes	4,728	0.36	0.22 – 0.58	
Blood transfusion before 1992				
No	23,915	1.08	0.96 – 1.22	< 0.001
Yes	1,226	7.34	6.01 – 8.94	
Abortion before 1990				
No	20,629	1.11	0.98 – 1.27	< 0.001
Yes	4,512	2.65	2.21 – 3.15	
Multiple surgeries				
No	19,025	1.12	0.98 – 1.28	< 0.001
Yes	6,116	2.65	1.88 – 2.62	
Multiple hospitalization				
No	17,817	0.98	0.84 – 1.13	< 0.001
Yes	7,324	2.39	2.06 – 2.77	
Multiple stomatological interventions				
No	11,703	0.97	0.81 – 1.17	< 0.001
Yes	13,438	1.75	1.54 – 1.98	
Hemodialysis				
No	25,005	1.37	1.23 – 1.52	0.003
Yes	136	4.41	1.99 – 9.50	
Car / work accidents requiring prolonged hospitalization				
No	24,064	1.35	1.22 – 1.51	0.032
Yes	1,077	2.14	1.42 – 3.19	
Intravenous drugs				
No	25,085	1.36	1.22 – 1.51	< 0.001
Yes	56	16.07	8.53 – 28.23	
Sexual contacts with multiple / unknown partners				
No	23,465	1.37	1.23 – 1.52	0.306
Yes	1,676	1.67	1.16 – 2.41	
Sharing personal hygiene objects				
No	19,693	1.26	1.12 – 1.43	0.001
Yes	5,448	1.84	1.51 – 2.23	
Tatooing / piercing				
No	23,270	1.34	1.20 – 1.49	0.014
Yes	1,871	2.03	1.48 – 2.78	

HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; LC: liver cirrhosis; HCC: hepatocellular carcinoma

All HCV positive persons were scheduled for further evaluation in a tertiary gastroenterology/hepatology center in their city, in order to be linked to care. The patients that presented for the staging of HCV infection and were detected with a positive HCV viral load from the total tested and detected HCV antibody positive were as follows: 80.2% in Bucharest, 66.6% in Cluj-Napoca, 86% in Iasi and 81.5% in Timisoara. Table 1.III shows the prevalence of HCV infection according to the risk factors identified by the addressed questionnaire. Only one risk factor (sexual contact with multiple/unknown partners) showed no association with HCV seropositivity.

The prevalence of anti-HCV antibodies increased with the number of risk factors present for one subject: if a patient had ≤1 risk factors, the HCV prevalence was 0.49%; if a patient had 2-3 risk factors, the HCV prevalence was 1.33%, increasing to 2.56% in cases where a patient had 4-5 risk factors or 6.48% in cases where a patient had ≥6 risk factors ($p<0.001$). When

taking into account the number of risk factors (4-17 risk factors), the regression analysis revealed a positive association between the presence of anti-HCV antibodies and female gender ($p<0.001$), rural area of residence ($p<0.001$) and advanced age ($p<0.001$), as well as a negative association with the education level ($p<0.001$). Table I.IV depicts the OR resulted from multiple logistic regression for potential risk factors of HCV infection.

Table 1.IV. Association between chronic-HCV infection and a risk factor identified in the questionnaire

Risk factors (answers from the questionnaire)	OR	95% CI	p (Z test)
Previous known KBV/HDV	0.73	0.40 – 1.33	0.306
Known HCV (+) family members	2.21	1.48 – 3.30	0.0001
Known HBV / HDV (+) family members	1.30	0.80 – 2.12	0.287
Decreased relatives with LC / HCC	0.94	0.66 – 1.34	0.729
Professional exposure to blood products	0.24	0.14 – 0.41	0.0001
Blood transfusion before 1992	3.15	2.28 – 4.36	0.00014
Abortion before 1990	1.38	1.05 – 1.80	0.020
Multiple surgeries	0.85	0.62 – 1.17	0.330
Multiple hospitalization	1.37	1.01 – 1.87	0.045
Multiple stomatological interventions	1.14	0.88 – 1.48	0.306
Hemodialysis	0.30	0.09 – 1.05	0.060
Car / work accidents requiring prolonged hospitalization	0.71	0.42 – 1.21	0.204
Intravenous drugs	3.70	1.19 – 11.55	0.024
Sexual contacts with multiple / unknown partners	0.92	0.57 – 1.47	0.718
Sharing personal hygiene objects	1.49	1.14 – 1.95	0.004
Tatooing / piercing	1.26	0.84 – 1.91	0.265

Discussion. Hepatitis C is a systemic disease with hepatic and extrahepatic manifestations resulting in increased morbidity and mortality in HCV-infected patients compared to cured or uninfected individuals. Launched in 2016 by the World Health Organization (WHO) and worldwide known, the Global Sectorial Strategy of Health for the elimination of Viral Hepatitis as a threat for the health status of the population by 2030 has ambitious objectives: decrease of the incidence of viral hepatitis by 90%, diagnosis of 90% of the infected people, access to therapy to 80% of the diagnosed and eligible persons, reduction with 65% of the liver mortality through integrated actions of awareness, testing and access to treatment (WHO, 2016).

Also, the launch of the Elimination Manifesto on 17 February 2016 provided a starting point for action to make HCV and its elimination in Europe an explicit public health priority, to ensure that patients, civil society groups and other relevant stakeholders will be directly involved in developing and implementing HCV elimination strategies (Papatheodoridis et al., 2018).

A key challenge to HCV elimination in Europe is the lack of reliable estimates of the burden of disease. Knowing the true burden of disease and the profile of those infected is necessary to design programs and policies to scale-up prevention and treatment (Hahné et al., 2013).

Today, we are fighting with an important public health threat, COVID-19, which certainly needs special attention; however, we should not neglect medical care of other viruses and diseases such as HCV/HBV/the human immunodeficiency virus infection (HIV). Quarantine and social distancing for COVID-19 can affect diagnosis, treatment and harm reduction programs. Increasing people's awareness plays an important role in viral hepatitis elimination programs leading to more case findings (Karimi-Sari et al., 2017; Hagan et al., 2019).

Thus, we consider this paper a step forward in our public health achievements for viral hepatitis elimination until 2030 in Romania, in this current difficult situation of the ongoing pandemic.

The success of this ambitious goal, at least in countries such as ours, is possible only by fragmentation in various strategies/campaigns of micro-elimination (at the national, institutional level, different target populations). However, this approach is used also in wealthy countries with very low prevalence such as Belgium or the Netherlands (Kracht et al., 2018; Soholm et al., 2019; Busschots et al., 2020). This concept targets specific population subgroups: children (under the age of 15 years), HCV/HIV-coinfected persons, birth cohorts, hemodialysis patients, those diagnosed with haemophilia, men who have sex with men, migrants, people with advanced liver disease, people who inject drugs, prisoners, and transplant recipients (Lazarus et al., 2018; Hagan et al., 2019; Hollande, 2020). In Romania, besides the already mentioned categories, we should include as target populations: people with the intrafamilial transmission of HCV, transfusions and abortions during the communist era, subjects with professional exposure to blood products and multiple hospitalizations. Also females, with advanced age, with a lower level of education and unemployed from rural areas were more susceptible to have HCV chronic infection.

These results are in line with our previous research (Gheorghe et al., 2010). However, the overall prevalence was significantly lower (1.39% vs 3.23% than previously reported). This trend indicates a cumulative risk of HCV infection over time, suggesting at the same time a cohort phenomenon with reduced transmission in recent years due to continuous improvement in healthcare conditions. The same aspects with decreased prevalence were noted in Italy (Cozzolongo, 2009) and Spain (Rodríguez-Tajes et al., 2020). A Polish study (Piekarska and Berkan-Kawińska A, 2018) reported the highest ratio of positive anti-HCV results in the group of young women aged <35 years with a positive history of at least one hospitalization (5.5%).

This proportion was significantly higher compared to the group of patients with arterial hypertension (1.2%) and patients with diabetes mellitus (1.06%), also hospitalized at least once. Given the obtained results it seems reasonable to look for new risk groups of HCV infection such as hospitalized persons to increase efficacy the of screening and consecutively of micro-elimination. The higher rural prevalence of HCV infection in Romania can be explained by the aging population in rural communities and a cohort effect, as well as by their hygiene conditions, lifestyle and mentalities which limit access to medical facilities. Due to these real situations in Romania, our next screening project that will take place from 2020 to 2024 will target this kind of vulnerable population. The same results were reported in rural areas in Italy almost 20 years ago, with an HCV positivity prevalence rate of 22.4/100, with an increasing age trend, with positive predominance among females; data from this study showed the effects of the inappropriate use of medications or surgery practices on the population (Raffaele et al., 2001).

Other identified risk factors for HCV increased prevalence are noted also in Southern Italy: abuse of intravenous drugs among people <60 years old, a history of tattooing/piercing, a history of dental surgery or surgical interventions, at least one previous blood transfusion. However, the high number of migrants from Romania to Italy can account for the rather similar risk factors in areas with high HCV prevalence. Removing HCV treatment reimbursement restrictions in many countries was a big step forward to HCV elimination. In Romania this happened only this year, but in other countries in Europe it occurred much earlier (Marshall et al., 2018; Busschots et al., 2020). Another challenge is access to DAAs therapy, especially for people from rural areas. At present, it can be prescribed and initiated only by a gastroenterologist and is available only in certain big towns. If in the future, DAAs therapy could be prescribed by other healthcare professionals and would be available in local pharmacies as well, treatment access would improve and patients would be able to receive their medication more conveniently. Consistent with our findings, HCV prevalence has increased with the rising burden of risk factors as shown also in the studies from France or Canada (Cadranel et al., 2008; Vaux et al., 2015; Parmar et al., 2016; Buonomo et al., 2018; Reyes et al., 2019).

Elimination of hepatitis C worldwide appears plausible, with higher chances of success if micro-elimination strategies are adopted instead of macro-elimination strategies based on mass-screening. Our study was possible by the multidisciplinary team efforts that contributed to the testing of all admitted patients and subsequently the positive subjects were sent to treatment and care in Gastroenterology/Hepatology specialized tertiary centers. We propose this study as a possible approach to achieving HCV micro-elimination in hospitalized people. It brings up-to-date HCV prevalence data which will assist strategic micro-elimination planning that is currently lacking in Romania.

Conclusions. In this hospital-based screening micro-elimination program in Romania, HCV prevalence was lower (1.39%) than previously reported. In a national campaign aimed at improving case finding and increasing awareness of hepatitis C, we demonstrated how targeted and locally adapted HCV testing and treating interventions may be successful in rapidly achieving microelimination in high-risk patient populations. This is the first step towards a cost-effective screening in well-defined group of persons at risk and provides sufficient capacity to deliver access to HCV treatment and care in Romania, bringing our country closer to the achievement of the WHO objective.

1.3.3. Hepatitis C Virus Prevalence and Risk Factors in a Village in Northeastern Romania—A Population-Based Screening—The First Step to Viral Micro-Elimination

Background & Aim. Considering the results from previous study, we aimed to assess the prevalence of HCV in a rural population from Moldavia, the Romanian region with the highest prevalence of HCV (Gheorghe et al., 2010) and to link the population to antiviral treatment. In this integrated project of testing-diagnosis-treatment, we established as objectives the following: micro-elimination of HCV, prevention of advanced HCV liver disease, prevention of HCV transmission among the healthy population and updating the epidemiological data regarding HCV in this region.

Materials and methods. We conducted a single-center prospective study based on the strategy of a project designed to educate, test and treat HCV with the aim of eliminating HCV infection in all adults in a village in the Moldavia region in north-eastern Romania—a region considered to have a poor socio-economic level and with limited access to the healthcare system. Our project was carried out from 1 March 2019 to 28 February 2020. To achieve these objectives, it was necessary to include all stakeholders—the local government authorities, associations of healthcare providers and patients. A mobile team of gastroenterologists, residents and nurses from the Institute of Gastroenterology and Hepatology, “St Spiridon“ Hospital Iasi was created, undertaking community mobilization with the aid of the local leadership (mayor, village council, general practitioners, teachers and priest). The entire population of the village over the age of 18 was invited to be tested by direct door-to-door communication. In some particular situations, the testing was organized at a household level, in order to perform screening at the level of the whole village.

The screening was carried out using rapid diagnostic orientation tests for HCV diagnosis and was performed for all subjects. All patients with anti-HCV antibodies were referred to a tertiary gastroenterology and hepatology department to confirm the active infection, staging and treatment prescription (linkage-to care). All demographic data through a questionnaire (Table 1.V).

The study was approved by the National Ethics Committee, and written informed consent was obtained from each patient in accordance with the principles of the 1975 Declaration of Helsinki.

Statistical Analysis. The collected data were statistically analyzed using the SPSS 20.0 (Chicago, IL, USA) software. The prevalence of anti-HCV antibodies subjects was calculated

with a 95% confidence interval (CI). Groups were compared using the Chi-square test or Fisher's exact test for categorical variables and by the independent t Student test or Mann–Whitney U test for continuous variables (depending on data distribution). Most of the investigated variables were calculated using logistic regression and odds ratio (OR) together with the corresponding 95% CI. All statistical tests were two-tailed, with a p-value ≤ 0.05 considered statistically significant.

Table 1.V. Addressed questionnaire in screened subjects

Questions
Part 1 – Demographic data
1. Age: o 18-29 years, o 30-39 years, o 40-49 years, o 50-59 years, o 60-69 years, o 70-79 years, o 80-97 years
2. Sex
- male
- female
3. Residence
- rural
- urban
4. Etnicity
- romanians
- roma
- other
5. Educational status
- subjects without university studies
- subjects with university studies
6. Marital status
- widowed or divorced
- married or living together as a couple
7. Social status
- retired
- housewife
- employee
Part 2 – Risk factors for HCV infection (answer from the questionnaires)
1. Previous known HBV / HDV Yes/No
2. Known HCV (+) family members Yes/No
3. Professional exposure to blood products Yes/No
4. Blood transfusions before 1992 Yes/No
5. Abortion before 1990 Yes/No
6. Multiple surgeries Yes/No
7. Multiple dental interventions Yes/No
8. Hemodialysis Yes/No
9. Sexual contacts with multiple / unknown partners Yes/No
10. IV (intravenous) drugs Yes/No
11. Tatooing / piercing Yes/No
12. Sharing personal hygiene objects Yes/No

Results. Prevalence of HCV Infection. All the village inhabitants—in total, 3507 subjects—were invited to be screened by rapid diagnostic orientation tests. Of these, 2945 (84%) subjects signed the informed consent and were consequently tested and enrolled in the study. The prevalence of positive HCV antibodies in the rural population that presented for testing was 2.64% (Figure 1.4).

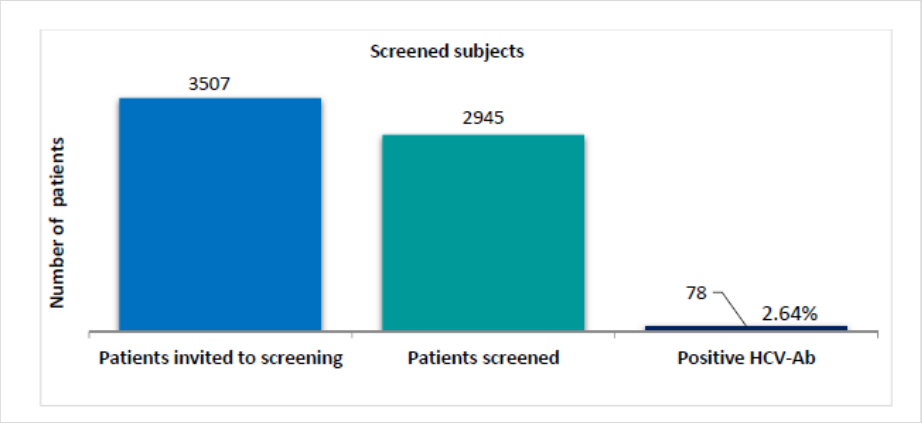

Fig. I.4. Results of screened participants and prevalence of positive HCV antibodies in the rural population

The study population consisted of 1973 females and 972 males. The mean age of subjects was 56.1 ± 14.5 years. Detailed baseline demographic characteristics of the study cohort are presented in Table 1.VI.

Table 1.VI. Baseline demographic characteristics

	Variable	Patients with Negative HCV Ab (n = 2867)	Patients with Positive HCV Ab (n = 78)	p-Value
Sex, n (%)				
	Male	947 (33)	25 (32.1)	0.815
	Female	1920 (67)	53 (67.9)	
Age, years, n (%)				
	18-29	284 (9.9)	1 (1.3)	0.163
	30-39	211 (7.4)	4 (5.1)	
	40-49	358 (12.5)	6 (7.7)	
	50-59	552 (19.2)	11 (14.1)	
	60-69	614 (21.4)	14 (17.9)	
	70-79	443 (15.5)	18 (23.1)	
	80-97	405 (14.1)	24 (30.8)	
Marital status, n (%)				
	Widowed or divorced	353 (12.3)	67 (85.9)	0.037
	Married or living together as a couple	2514 (87.7)	11 (14.1)	
Social status, n (%)				
	Retired persons	1277 (44.5)	44 (56.4)	0.26
	Housewives	810 (28.3)	28 (35.9)	
	Employees	780 (27.2)	6 (7.7)	
Educational status, n (%)				
	Subjects without university studies	2510 (87.5)	73 (93.6)	0.188
	Subjects with university studies	357 (12.5)	5 (6.4)	

Overall, the distribution of subjects according to gender was similar for both negative and positive HCV antibodies statuses. Regarding age groups, the prevalence of HCV antibodies was found to be higher in those between 80–97 years (30.8% versus 14.1%). Furthermore, the prevalence of HCV was higher in widowed subjects (85.9% versus 12.3%)

and lower in those married or living together as a couple (14.1% versus 87.7%). According to social and educational status, the prevalence of positive HCV Ab was lower in employees (7.7% versus 27.2%) and those with university studies (6.4% versus 12.5%).

Cascade of Care

All subjects with positive HCV antibodies (78, respectively 2,64%) were scheduled for further complete evaluation in a tertiary gastroenterology and hepatology center nearby in order to be linked to care. In total, 66 (85%) subjects were presented for evaluation, and 55 (83%) subjects had detectable HCV RNA. Of these, 54 (98%) patients completed antiviral treatment and 53 (99%) obtained SVR. The cascade of care is shown in Figure 1.5.

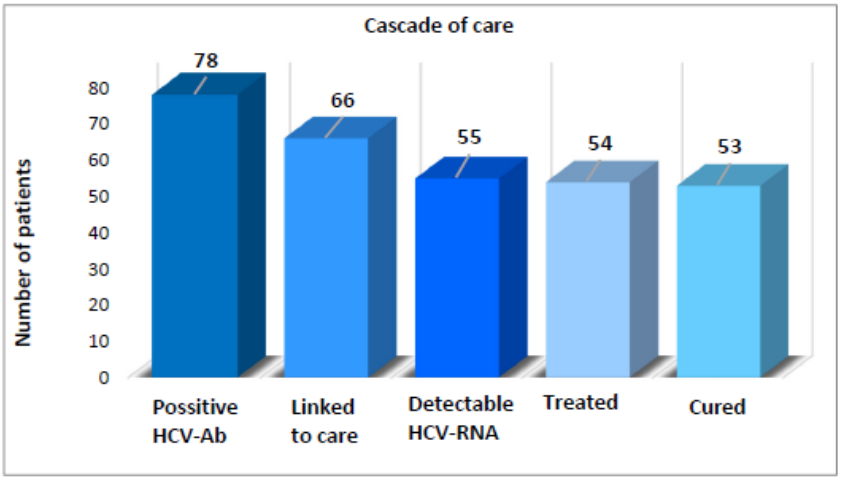

Fig. 1.5. Cascade of care step by step. Positive HCV Ab: Number of people estimated to have viremic HCV infection. Linked to care: Number of patients evaluated for treatment. Detectable HCV-RNA: Number of patients who received a diagnosis of viremic HCV infection. This number excludes patients who were cured of their infection or who had experienced the spontaneous clearance of their infection before 2019. Treated: Number of patients who initiated HCV treatment (all types of treatment, interferon-based regimens). Cured: Number of patients who obtained SVR.

Association between Risk Factors and Chronic HCV Infection

According to the addressed questionnaire, three (3.84%) patients were identified with a history of HBV/HDV, five (6.41%) had a history of HCV infection, eight (10.25%) individuals had undergone abortions before 1990, six (7.69%) had experienced multiple surgeries, four (5.12%) had blood transfusions before 1992, eleven (14.10%) patients had multiple dental interventions, two (2.56%) declared sexual contacts with multiple partners, one (1.28%) was using intravenous drugs, three (3.84%) patients had undergone tattooing/piercing procedures, eight (10.25%) shared personal hygiene objects, and no patients had professional exposure to blood products or hemodialysis (Table 1.VII). The main risk factors associated with chronic HCV infection were a family history of HCV (OR = 2.23, 95%CI = 1.37–3.5, $p < 0.0001$), blood transfusions performed before 1992 (OR = 3.21, 95%CI = 2.25–4.52, $p < 0.0001$), abortions conducted before 1990 (OR = 1.35, 95%CI = 1.02–1.9, $p = 0.023$), multiple surgical interventions (OR = 1.32, 95%CI = 1.05–1.72, $p = 0.038$) and sharing personal hygiene objects (OR = 1.45, 95%CI = 1.12–1.73, $p = 0.002$).

In our subjects, we observed that the main risk factors associated with chronic HCV infection were a family history of HCV (OR = 2.23, 95%CI = 1.37–3.5, $p < 0.0001$), blood transfusions performed before 1992 (OR = 3.21, 95%CI = 2.25–4.52, $p < 0.0001$), abortions conducted before 1990 (OR = 1.35, 95%CI = 1.02–1.9, $p = 0.023$), multiple surgical interventions (OR = 1.32, 95%CI = 1.05–1.72, $p = 0.038$) and sharing personal hygiene objects (OR = 1.45, 95%CI = 1.12–1.73, $p = 0.002$).

Table 1.VII. Risk factors associated with chronic HCV infection

Risk Factors	HCV Negative (N = 2867) n (%)	HCV Positive (N = 78) n (%)	OR	95% CI	p-Value
Known HBV/HDV	46 (1.60)	3 (3.84)	0.62	0.30–1.31	0.302
Known HCV (+) family members	47 (1.63)	5 (6.41)	2.23	1.37–3.50	0.0001
Professional exposure to blood products	78 (2.72)	0 (0.00)	0.25	0.11–0.53	0.0001
Abortion before 1990	141 (4.91)	8 (10.25)	1.35	1.02–1.90	0.023
Multiple surgeries	83 (2.89)	6 (7.69)	1.32	1.05–1.72	0.038
Blood transfusions before 1992	45 (1.56)	4 (5.12)	3.21	2.25–4.52	0.0001
Multiple dental interventions	67 (2.33)	11 (14.10)	1.12	0.67–1.45	0.303
Hemodialysis	33 (1.15)	0 (0.00)	0.34	0.06–1.03	0.062
Sexual contacts with multiple partners	133 (4.63)	2 (2.56)	0.88	0.52–1.38	0.615
Intravenous drugs	78 (2.72)	1 (1.28)	0.71	0.40–1.44	0.302
Tattooing/piercing	81 (2.82)	3 (3.84)	1.25	0.72–1.84	0.251
Sharing personal hygiene objects	103 (3.59)	8 (10.25)	1.45	1.12–1.73	0.002

OR, odds ratio; CI, confidence interval; HVB, hepatitis B virus

Discussion. The global elimination of HCV has become the ultimate endeavor and final objective since the introduction of DAAs. However, the simple availability of these drugs, which can reduce the burden of HCV infection, is not enough to achieve a real impact on morbidity and mortality, much less to target viral eradication (Maticic et al., 2020).

A more pragmatic approach is the concept of "micro-elimination", which involves the elimination of hepatitis C in defined segments of the risk population, as well as in geographical areas (regions, cities, villages) as a strategy to incrementally achieve national elimination (Gheorghe et al., 2020).

Thus, we consider this work as a small step forward in our public health achievements for the elimination of viral hepatitis by 2030 in Romania. The success of this ambitious goal, at least in countries such as Romania, is possible only by dividing it into different micro-elimination campaigns such as the project we have carried out at a sub-regional level in a population with difficult access to the healthcare system. In this screening micro-elimination program conducted in a sub-region of Romania, the prevalence of HCV was lower (2.64%) than previously reported (Gheorghe et al., 2020).

As far as we know, there have been no similar projects in Europe carried out in rural areas. Worldwide there are several HCV micro-elimination projects conducted at the community levels, an Egyptian project in which HCV micro-elimination was also initiated in rural areas had successful outcomes; 99.9% of patients completed the antiviral treatment and 97% achieved an SVR (Shiha et al., 2020). In our study, 98% of positive screened HCV patients received antiviral therapy, 100% completed the treatment and 99% achieved SVR.

The main risk factors identified in our subject for the increased prevalence of HCV are family members known to be positive for HCV and sharing personal hygiene items. Also, subjects with abortions or blood transfusions conducted before 1990, multiple surgical interventions and who shared personal hygiene items show the effects of the inappropriate use of medical or surgical practices on the population.

The data from this study show that a real challenge for people with positive HCV antibodies in this rural area was access to a tertiary gastroenterology/hepatology center located at a distance for further evaluations.

The elimination of hepatitis C worldwide is becoming a possibility, with higher chances of success if micro-elimination strategies based on mass screening are implemented.

A sustained effort on the part of all stakeholders is required, including governmental authorities at the national and local levels, associations of healthcare providers, patients, and representatives of at-risk populations. Before attempting nationwide elimination, breaking

down national elimination goals into smaller, achievable goals for individual population segments may be more realistic (Safreed-Harmon et al., 2019).

Conclusions. This micro-elimination project carried out in a rural area is the first in Romania and among a small number of such projects in the world. Micro-elimination involves fewer resources than large-scale country-level initiatives to eliminate HCV and can represent a boost for small victories that inspire large and ambitious efforts. The development of screening programs is crucial for the accessibility of treatment and the achievement of WHO objectives.

Our micro-elimination project showed a 2.64% HCV prevalence in rural areas, higher than reported by Gheorghe L, et al in the hospitalized population (Gheorghe et al., 2020). According to our findings, the HCV prevalence in Moldavia is 1.41%, much lower than that reported in another study from 2010, where the seroprevalence of HCV was higher compared to that in the Southern Region 4.06% vs. 3.26% ($p = 0.065$); the logistic regression analysis showed that the risk of having HCV is 1.26 times higher for a resident from the North-East of Romania compared to the one from the South of the country (Gheorghe et al., 2010).

The higher prevalence of HCV infection in patients from rural area in Moldavia, Romania could be explained by the aging population in rural communities, deficitary hygiene conditions, lifestyle and difficult access to a tertiary gastroenterology center.

1.4. NON -ALCOHOLIC FATTY LIVER DISEASE - THE NEXT CHALLENGE IN HEPATOLOGY

1.4.1. Introduction

NAFLD includes a wide range of manifestations, from simple hepatic steatosis (a benign/silent disease with fat accumulation in liver volume more than 5%) to steatohepatitis (NASH) (steatosis with inflammation, fibrosis with high potential for progression), to liver cirrhosis (and hepatocellular carcinoma), the final stage of any chronic liver disease (Rinella et al., 2014; Singh et al., 2015; Younossi et al., 2016).

NAFLD has become a frequent cause of chronic liver disease in our century, affecting one one-quarter adults worldwide (Younossi et al., 2016).

The global burden of NAFLD is rising in parallel with increasing rates of obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome (Williamson et al., 2011; Takalkar et al., 2018). Although it is estimated that one in three patients with NAFLD has non alcoholic steatohepatitis (NASH), the true prevalence of NASH is not known, certainly due to the asymptomatic course of the disease; that's meaning that millions of people worldwide are at risk of cirrhosis (Younossi et al., 2016). Moreover, NAFLD is also a growing risk factor for HCC and a leading indication for liver transplantation (Wong et al., 2015; Younossi et al, 2018) and of HCC-related liver transplantation (Younossi et al., 2019).

Statistical analyses showed that in Europe, in 2030, a timepoint we established to eradicate the hepatitis C virus infection, it is estimated that the highest prevalence of NAFLD will be in Italy (29.5%) and the lowest (23.6%) in France (Younossi et al., 2019).

1.4.2. The Prevalence of Liver Steatosis and Fibrosis in Apparently Healthy Romanian Medical Students

Background & Aim. There are several studies regarding the increasing prevalence of NAFLD in young adults, which parallels the high rates of obesity and metabolic syndrome in this age group (Mrad et al., 2017; Doycheva et al., 2017). The term "young adult" is very familiar to oncologists and refers to a population of patients starting from the age of 20 years

without being able to set the upper age limit in clinical practice (Barr et al., 2016). Among these patients, some present a number of risk factors for developing NAFLD, such as: obesity, T2DM, unhealthy lifestyle, smoking habits, and male sex. The interplay between these factors on a background of genetic predisposition may contribute to the installation of NASH (Doycheva et al., 2017). Vibration-Controlled Transient Elastography (VCTE) is considered the optimal non-invasive method for assessing LF, recommended by guidelines over the years for fibrosis evaluation, especially in chronic viral hepatitis (The European Association for the Study of the Liver -EASL) 2015, WHO 2016).

In addition, in recent years, the implementation of Controlled Attenuation Parameter (CAP - that reflects the fat impedance in the liver) in FibroScan® (Echosens, Paris, France) devices has allowed the concomitant evaluation of hepatic fibrosis and steatosis (Karlas et al., 2017; Chalasani et al., 2018).

Herein, we aimed to evaluate the prevalence of steatosis and fibrosis in apparently healthy Romanian medical students by VCTE and CAP score. We also researched risk factors associated with hepatic steatosis and fibrosis in this population group.

Materials and Methods. This study population consisted of apparently healthy 3rd and 5th-year medical students, with a high level of education, from "Grigore T. Popa" University of Medicine and Pharmacy Iasi, evaluated between February and June 2021. Demographic data, personal history, clinical examination, and data obtained from their general practitioner were recorded along with anthropometric and FibroScan assessments. Eligibility criteria were the absence of significant alcohol consumption (<20 g/day in women, <30 g/day in men) and of a history of chronic liver disease. Participants with unreliable transient elastography examination (<10 valid measurements with an interquartile range/median (IRQ/M) ratio >30%) were excluded. For subjects with a liver stiffness measurement (LSM value) ≥7.2 kPa on VCTE examination, laboratory data were collected. This study was approved by the Ethics Committee of our university and was conducted according to the principles of the Declaration of Helsinki. Each student signed a written informed consent.

LSM and CAP Assessment. All students were evaluated using FibroScan® 502 Touch (Echosens, Paris, France) by one experienced physician with more than 1000 explorations performed before, using one single examination on each subject, following procedure instructions. LSM were expressed in kilopascals (kPa), with the following cut-offs for LF: ± 5.5 kPa—F0 (without fibrosis) 5.6 kPa—F1 (mild), 7.2 kPa—F2 (significant), 9.5 kPa—F3 (advanced), and 12.5 kPa—F4 (cirrhosis). Liver steatosis measured by CAP was expressed in decibels/meter (dB/m), and steatosis degrees were S1 (mild)—248 dB/m, S2 (moderate)—268 dB/m, and S3 (severe)—280 dB/m.

Anthropometric Measurements. Height and weight measurements were performed using a height meter and the weight scale. Overweight (± 25 kg/m2) and obesity (>30 kg/m2) were established using cut-off values defined by the WHO, while waist-to-height ratio (WtHR) is defined by dividing waist circumference (cm) to height (cm), with a settled value ± 0.50 (McCarthy et al. 2006).

Statistical analyses were performed using SPSS software version 22.0 (IBM SPP Inc., Chicago, IL, USA). Qualitative data were expressed as numbers (percentage), while quantitative variables were expressed as means ± standard deviation (SD). The Kolmogorov–Smirnov test was used for distribution analysis, continuing with the Student's t-test, Mann–Whitney U, or chi-square test that was considered appropriate for comparing group variables. The association between two variables was made by utilizing the Pearson correlation coefficient (r). Two-tailed p-values of <0.05 were considered statistically significant.

Results. *Participants Characteristics.* A total of 505 subjects were invited to participate in this study, 439 of which were evaluated by VCTE and CAP. 13 participants were excluded due to unreliable measurements (10 cases) and examination failure without any measurements (3 cases). Four hundred and twenty-six medical students who met the admission standards were included in the final analysis. All baseline characteristics are summarized in Table 1.8.

The prevalence of overweight, obesity, and abdominal obesity was 14.8%, 3.5%, and 7.5%, respectively. Most of the participants were in the 21-year-old group, with a predominance of female gender (67.8% females, mean age 22.22 ± 1.7 years, and body mass index (BMI) 22.59 ± 3.34 kg/m^2).

Table 1.8. The characteristics of the overall participants included in study

	Overall Cohort n, 426	**Men n, 137**	**Women n, 289**	**p-Value**
Age (years)	22.22 ± 1.7	22.45 ± 1.8	22.11 ± 1.6	0.144
Females, n (%)	289 (67.8)	-	-	
Weight (kg)	65.84 ± 13.37	74.09 ± 13.29	61.95 ± 11.56	<0.001
Height (cm)	170 ± 8.56	176 ± 10.2	167 ± 10.7	<0.001
Body mass index (kg/m2)	22.59 ± 3.34	23.71 ± 3.33	22.07 ± 3.22	<0.001
Waist circumference (cm)	73.7 ± 10.29	78.79 ± 11.35	71.31 ± 8.82	<0.001
Abdominal obesity, n (%)	32 (7.5%)	17 (12.4%)	13 (4.5%)	<0.001
Waist-to-height ratio	0.427 ± 0.06	0.442 ± 0.06	0.42 ± 0.05	0.159
Non-overweight, n (%)	348 (81.7)	102 (74.5)	246 (85.1)	0.046
Overweight, n (%)	63 (14.8)	27 (19.7)	36 (12.5)	0.004
Obese, n (%)	15 (3.5)	8 (5.8)	7 (2.4)	0.037
Liver steatosis, n (%)	74 (17.4)	39 (28.5)	35 (12.1)	0.011
Steatosis degree, n (%)				0.026
0	352 (82.6)	98 (71.5)	254 (87.9)	
1	32 (7.5)	18 (13.1)	14 (4.8)	
2	13 (3.1)	5 (3.7)	8 (2.8)	
3	29 (6.8)	16 (11.7)	13 (4.5)	
Fibrosis stage, n (%)				0.186
0	277 (65)	79 (57.6)	198 (68.5)	
1	136 (31.9)	50 (36.5)	86 (29.8)	
2	10 (2.4)	6 (4.4)	4 (1.4)	
3	3 (0.7)	2 (1.5)	1 (0.3)	
CAP, dB/m	215.76 ± 48.38	234.49 ± 47.38	206.95 ± 46.42	<0.001
LSM, kPa	5.29 ± 1.35	5.36 ± 1.2	5.26 ± 1.42	0.582
M-probe, n (%)	402 (94.4)	128 (93.4)	274 (94.8)	0.410
XL-probe, n (%)	24 (5.6)	9 (6.7)	15 (5.2)	0.372

n-number of subjects; CAP, controlled attenuation parameter; LSM, liver stiffness measurement

Men were heavier (74.09 ± 13.29 kg vs. 61.95 ± 11.56 kg, $p < 0.001$), taller (176 ± 10.2 cm vs. 167 ± 10.7 cm, $p < 0.001$), with a greater proportion of overweight (19.7% vs. 12.5%, $p = 0.004$), obesity (5.8%vs. 2.4%, $p = 0.037$), and abdominal obesity (12.4%vs 4.5%, $p < 0.001$) than women.

The prevalence of hepatic steatosis among all students was 17.4%, with a mean CAP of 215.76 ± 48.38 dB/m; 32 (43.2%) of them had S1, and 42 (56.8%) had significant steatosis (S2-S3) with a CAP score above 268 dB/m. The proportion of male students among steatosis degrees was higher compared to women ($p = 0.026$), with an increased CAP value (234.49 ± 47.38 dB/m vs. 206.95 ± 46.42 dB/m, $p < 0.001$). Regarding the prevalence of LF, the majority (277 students, 65%) had no LF, while 136 (31.9%) participants had F1, 10 (2.4%) had F2, 3 (0.7%) had F3, and no one was found with F4 LF; the mean LSM of 5.29 kPa ± 1.35. Subjects with a LF ± F2 were predominantly males (61.5%) with a mean BMI of 24.58 ± 3.41 kg/m2 and a WtHR 0.462 ± 0.07. The characteristics of patients with LF ≥ F2 are presented in Table 1.9.

Table 1.9. Increased clinical and laboratory parameters in patients with liver fibrosis ≥ F2

	Subjects, n = 13	Increased, n (%)
Age (years)	22.7 ± 1.5	-
Males, n (%)	8 (61.5)	-
Body mass index (kg/m2)	24.58 ± 3.41	8 (61.5)
Waist-to-height-ratio	0.462 ± 0.07	5 (38.5)
Platelet count (G/L)	287 ± 72.45	0 (0)
ALT (IU/L)	24.7 ± 14.9	3 (23.1)
AST (IU/L)	26.3 ± 11.4	4 (30.7)
GGT (IU/L)	25.1 ± 16.6	2 (15.3)
ALP (IU/L)	62.7 ± 20.2	0 (0)
Total bilirubin (mg/dL)	0.68 ± 0.25	0 (0)
Fasting glucose (mg/dL)	88.3 ± 17.1	6 (46.1)
Total cholesterol (mg/dL)	208.5 ± 38.3	5 (38.5)
Triglycerides (mg/dL)	131.6 ± 52.9	7 (53.8)
LDL-c (mg/dL)	112.1 ± 26.6	3 (23.1)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; LDL-c low density lipoprotein cholesterol. Increased values: BMI > 25 kg/m2; WtHR > 0.5; ALT > 35 IU/L; AST > 35 IU/L; GGT > 40 IU/L; ALP > 140 IU/L; Total bilirubin > 1 mg/dL; Fasting glucose > 100 mg/dL; Total cholesterol > 200 mg/dL; Triglycerides > 150 mg/dL; LDL-c > 130 mg/dL

Participants Characteristics according to Absence or Presence of Liver Steatosis. The participants included in the study that were diagnosed with liver steatosis were predominantly males (p = 0.031), with an increased weight (p < 0.001), BMI (p < 0.001), WC (p < 0.001), and WtHR (p < 0.001) (Table 1.10). However, the proportion of overweight (40.5% vs. 9.4%, p < 0.001) and obese (9.5% vs. 2.3%, p < 0.001) students was significantly higher among the liver steatosis group, of whom 18.9% had abdominal obesity. Regarding LF stages, 32 (43.2%) of students had mild fibrosis (F1), 5 (6.8%) had significant fibrosis (F2), and 3 (4.1%) had advanced LF (F3), with an increased LSM value (p = 0.027) compared with those without hepatic steatosis, consisting of 104 (29.6%) with F1, 5 (1.4%) with F2, and none with advanced LF (p = 0.024).

Table 1.10. Baseline characteristics of participants according to the presence of liver steatosis

	No Hepatic Steatosis n, 352	Hepatic Steatosis n, 74	p-Value
Age (years)	22.18 ± 1.61	22.36 ± 1.73	0.565
Males, n (%)	98 (27.8)	39 (52.7)	0.031
Weight (kg)	63.14 ± 11.37	75.09 ± 16.06	<0.001
Height (cm)	170 ± 10.5	171 ± 10.8	0.061
Body mass index (kg/m2)	22.14 ± 3.04	24.89 ± 3.91	<0.001
Waist circumference (cm)	71.9 ± 8.82	81.23 ± 12.94	<0.001
Abdominal obesity, n (%)	18 (5.1%)	14 (18.9%)	<0.001
Waist-to-height ratio	0.418 ± 0.05	0.482 ± 0.09	<0.001
Non-overweight, n (%)	311 (88.3)	37 (50)	0.029
Overweight, n (%)	33 (9.4)	30 (40.5)	<0.001
Obese, n (%)	8 (2.3)	7 (9.5)	<0.001
Fibrosis stage, n (%)			0.024
0	243 (69)	34 (45.9)	
1	104 (29.6)	32 (43.2)	
2	5 (1.4)	5 (6.8)	
3	0 (0)	3 (4.1)	
CAP, dB/m	199.16 ± 35.39	280.41 ± 38.95	<0.001
LSM, kPa	5.23 ± 1.35	5.61 ± 1.28	0.027
M-probe, n (%)	341 (96.9)	61 (82.4)	0.244
XL-probe, n (%)	11 (3.1)	13 (17.6)	<0.001

CAP, controlled attenuation parameter; LSM, liver stiffness measurement

Correlation between Anthropometric Parameters, CAP and LSM. Overall, we found a significant correlation between CAP and WtHR ($r = 0.36$, $p < 0.001$) BMI ($r = 0.34$, $p < 0.001$), weight ($r = 0.34$, $p < 0.001$), and waist circumference ($r = 0.33$, $p < 0.001$). Regarding LF expressed by LSM, only WtHR ($r = 0.13$, $p = 0.040$), BMI ($r = 0.21$, $p = 0.001$), and waist circumference ($r = 0.14$, $p = 0.024$) maintained a significant correlation.

Discussion. NAFLD is a very frequent cause of chronic liver disease, affecting approximately 25% of the world population; NASH and related significant fibrosis are the greatest predictors of high mortality, liver cirrhosis, and HCC (Younossi et al., 2018).

The most important study that analyses the prevalence of NAFLD in young adults was conducted by Mrad et al. in the United States of America, on a population aged from 18 to 35 years, which showed that the prevalence of NAFLD has risen 2.5 times in the last three decades, affecting 25% of the young adults nowadays (Mrad et al., 2017). Moreover, the authors concluded that the implementation of a screening program is needed in this age group to prevent the development of cirrhosis and its complications.

To the best of our knowledge, this is the first study on the prevalence of NAFLD and LF among Romanian medical students. In our study, approximately one in five students, who were apparently healthy, had hepatic steatosis, and one in thirty-three had significant LF (≥F2). In addition, in line with the current literature, we have demonstrated that male gender, BMI, waist circumference, and waist-to-height ratio were the main risk factors associated with hepatic fat accumulation.

Most of the students included in our study had no hepatic steatosis and our results are quite similar to those reported by recent studies. In similar research, Kaya et al. reported a 23.2% NAFLD prevalence in a group of 112 medical students with a mean CAP value of 205.6 ± 43.8 dB/m (Kaya et al., 2016). Moreover, Abeysekara et al. conducted a study in Great Britain, which included only apparently healthy young adults, and found that the prevalence of hepatic steatosis was 20.7%, with significant steatosis approximately two-thirds of the patients diagnosed with steatosis based on VCTE (Abeysekara et al., 2020).

In our group of subjects we found that the male sex, high BMI, waist circumference, WtHR, and an increased LSM value ($p = 0.027$) are independently risk factors associated with high values of CAP score and our data are in accordance with other recent studies (Doycheva et al., 2017; Abeysekara et al., 2020).

Most of participants had no LF or had only a mild form. Shaheen et al. found a high prevalence of NAFLD among Egyptian young adults (47.5% had variable stages of steatosis) and 56.7% had fibrosis (Shaheen et al., 2019). When compared to our findings, these results seem to be significantly different, and a possible reason could be the demographic contrast between the studied cohorts, considering the increased incidence of obesity and metabolic syndrome in the Egyptian population.

Nevertheless, the prospective design of this study countervails these drawbacks, as it includes a large series of asymptomatic patients with a high level of education. An advanced imaging technique, such as VCTE with CAP, was used for establishing the diagnosis of LF and steatosis, methods validated and correlated with histological findings based on LB in NAFLD patients (Vuppalanchi et al., 2018).

Conclusions. In summary, our findings show that the prevalence of steatosis and significant fibrosis among our cohort of apparently healthy medical students is low. In addition, we identified that overweight and obesity were not very common, but high BMI, WtHR, and WC values are associated risk factors for liver steatosis, as well as fibrosis. Therefore, the growing obesity epidemic can be avoided by a multidisciplinary approach to include lifestyle changes with special attention to regular physical exercise. Furthermore, individualized screening strategies should be established for significant LF and steatosis according to anthropometric indices.

1.4.3. Liver Steatosis and Fibrosis in Patients with Nonalcoholic Fatty Liver Disease

Background & Aim. The most important predictor for poor outcomes in chronic liver disease, including overall mortality rates, is the LF stage. Therefore, identifying patients with severe LF is mandatory, and this could be achieved by using a non-invasive method such as VCTE, with high acceptability among patients, as is a noninvasive method (Ekstedt et al., 2015). This prospective study aimed to confirm the diagnosis of NAFLD and measure the degree of the hepatic steatosis as well as the stage of LF in patients referred by general practitioners and colleagues in other specialties with clinically suspected or US diagnosed NAFLD, using VCTE and CAP mode. In addition, we aimed to evaluate the risk factors associated with liver steatosis and fibrosis.

Materials and Methods. Two hundred four patients with clinically suspected and/or US diagnosed NAFLD, referred to our clinic by general practitioners and colleagues in other specialties, were prospectively enrolled from September 2019 to February 2020 at the Gastroenterology and Hepatology Institute, in Iasi, Romania. The inclusion criteria were 1. age ±18 years; 2. signed informed consent. Exclusion criteria: 1. excessive daily alcohol consumption (defined as >30 g/day for males and >20 g/day for females); 2. history of other causes of chronic liver disease; 3. other causes of hepatic steatosis (steatogenic medication - chemotherapy, corticosteroids, tamoxifen, methotrexate, amiodarone, estrogen); 4. aspartate aminotransferase /alanine aminotransferase more than 10 times the upper limit of normal, or total bilirubin level more than 5 mg/dL; 5. pregnant women. The study was performed according to the guidelines of the Declaration of Helsinki and approved by our Institution's Ethics Committee.

Clinical and Laboratory Assessment. Demographic and clinical details were collected: gender, age, body mass index (BMI), the presence of T2DM (taking anti-diabetic drugs or fasting glucose >126 mg/dL), arterial hypertension (antihypertensive drugs use, systolic blood pressure >140 mmHg or diastolic blood pressure > 90 mmHg). Fasting blood tests were collected at baseline. For each patient, we calculated the most commonly used surrogate serum fibrosis markers using standardized equations (aspartate aminotransferase to platelet ratio index (APRI), fibrosis-4 index (FIB-4 index), and NAFLD fibrosis score (NFS).

VCTE Examination. All patients were examined using FibroScan® 502 Touch (Echosens, Paris, France) by an experienced physician. LSM results were expressed in kilopascals (kPa) ranging from 1.5 to 75 kPa, with cut-off staging values as follows: F0 (without <5.5 kPa, F1 (mild -5.6–7.1 kPa), F2 (significant - 7.2–9.4 kPa), F3 (advanced fibrosis - 9.5–12.4 kPa), and F4 (cirrhosis >12.5 kPa). CAP was expressed in decibels/meter (dB/m) ranging from 100 to 400 dB/m with values indicating S0 (without - <237 dB/m), S1 (mild – >237 dB/m),), S2 (moderate- >259 dB/m), S3 (severe - >291 dB/m) steatosis.

Statistical Analysis. The data were analyzed using IBMSPSS, Version 22.0 (IBMSPSS Inc, Chicago, IL, USA). Statistics for categorical variables are expressed as numbers (percentage). Baseline characteristics and clinical variables were expressed as mean ± standard deviation, if normally distributed, or as median (25th and 75th percentiles), if not normally distributed. Distribution analysis was performed using the Kolmogorov–Smirnov test. An unpaired t-test was used for comparison of continuous variables between groups for normally distributed data, and Mann–Whitney or the Kruskal–Wallis test was used to analyze skewed data. One-way ANOVA was considered appropriate to assess the difference in LSM values according to the degree of steatosis. Univariate linear regression was first performed to identify the factors that may influence the CAP and LSM values, followed by multivariate linear regression using only the significant factors. Pearson correlation coefficient (r) was used for establishing the association between two variables. A p-value of < 0.05 (two-tailed) was considered statistically significant. Only complete data sets were analyzed.

Results. *Patient Characteristics.* 204 patients who were clinically suspected or diagnosed with NAFLD based on US were enrolled and evaluated using VCTE with CAP. 23 (11.3%) patients were excluded (unreliable measurements in 18 of them, and examination failure without measurement values in 5 patients). Finally, 181 patients with reliable acquisitions were included (53% females with a mean age of 57.62 ± 11.8 years), 96 (52.5%) of whom were examined with the M probe and 85 (47.5%) with the XL probe, with a median CAP and LSM values in all measurements of 293 (245.5–339) dB/m and 6.1 (4.8–8.3) kPa, respectively. Obesity, T2DM, and hypertension were present in 87 (48%), 39 (21.5%), and 54 (29.8%) of patients, respectively. Baseline characteristics of all patients with reliable acquisitions included in the final analysis are summarized in Table 1.11.

Out of 181 patients analyzed, 171 (94.5%) had different grades of steatosis. These patients were older (mean age 59.19 ≥ 11.05 years, $p < 0.001$), with an increased BMI (30.37 ≥ 4.56, kg/m2, $p < 0.001$), and presented T2DM ($p < 0.001$) and hypertension ($p < 0.001$) in a higher proportion compared to those without steatosis. According to serological parameters, patients with steatosis presented a higher level of fasting plasma glucose ($p = 0.024$), ferritin ($p = 0.047$), C-reactive protein (CRP) ($p = 0.048$), total cholesterol ($p = 0.031$), low-density lipoprotein cholesterol ($p = 0.009$), and serum uric acid ($p < 0.001$), and a low level of high-density lipoprotein cholesterol ($p = 0.006$). Furthermore, they also had increased LSM values ($p = 0.049$), FIB-4 index ($p = 0.027$), NFS ($p = 0.007$), and CAP score ($p < 0.001$) (Table 1.11).

Table 1.11. Baseline characteristics of patients according to the presence of hepatic steatosis (≥S1)

	Overall Cohort	**No Steatosis**	**Any Steatosis (≥S1)**	**p-Value**
Patients characteristics	n = 181	n, (%) = 10 (5.5)	n, (%) = 171 (94.5)	
Gender (female), n (%)	96 (53)	4 (40)	92 (54)	0.184
Age, y	57.62 ± 11.8	51.08 ± 12.74	59.19 ± 11.05	<0.001
BMI (kg/m^2)	29.48 ± 4.85	25.76 ± 4.30	30.37 ± 4.56	<0.001
BMI ≥ 30 kg/m2, n (%)	87 (48)	2 (20)	86 (50.3)	<0.001
Diabetes, n (%)	39 (21.5)	1 (10)	38 (25.9)	<0.001
Hypertension, n (%)	54 (29.8)	2 (20)	52 (30.4)	<0.001
Platelet count (G/L)	251.67 ± 81.18	256.42 ± 131.39	250.53 ± 64.23	0.798
ALT (IU/L)	40.2 ± 41.29	29.75 ± 19.34	42.72 ± 44.71	0.136
AST (IU/L)	31.77 ± 22.61	25.21 ± 11.15	33.35 ± 23.37	0.079
GGT (IU/L)	58.89 ± 67.91	41.64 ± 31.07	57.33 ± 68.03	0.237
ALP (IU/L)	80.70 ± 36.92	76.67 ± 30.65	79.37 ± 35.01	0.709
Total bilirubin (mg/dL)	0.70 ± 0.38	0.75 ± 0.39	0.69 ± 0.38	0.465
Albumin (g/dL)	4.56 ± 0.38	4.53 ± 0.44	4.57 ± 0.36	0.559
Creatinine (mg/dL)	0.83 ± 0.13	0.814 ± 0.13	0.834 ± 0.13	0.505
Urea (mg/dL)	36.56 ± 10.81	34.40 ± 37.08	37.08 ± 11.22	0.188
Fasting glucose (mg/dL)	111.37 ± 43.77	96.37 ± 18.86	114.96 ± 47.19	0.024
Ferritin (ng/mL)	146.12 ± 114.98	111.54 ± 81	154.41 ± 120.47	0.047
CRP (mg/dL)	0.62 ± 2.27	1.38 ± 5.06	0.437 ± 0.482	0.048
Total cholesterol (mg/dL)	211.68 ± 55.04	191.61 ± 67.19	216.52 ± 50.85	0.031
Triglycerides (mg/dL)	148 ± 98.78	124.03 ± 113.49	153.78 ± 94.53	0.153
LDL-c (mg/dL)	125.34 ± 47.02	104.5 ± 45.59	130.37 ± 46.15	0.009
HDL-c (mg/dL)	45.06 ± 13.90	51.5 ± 13.47	43.5 ± 13.6	0.006
Serum uric acid (mg/dL)	5.21 ± 1.63	4.25 ±1.7	5.44 ± 1.53	<0.001
Alpha-fetoprotein (ng/mL)	3.84 ± 1.73	3.73 ± 1.13	3.87 ± 1.85	0.703
LSM (kPa)	6.1 (4.8–8.3)	5 (4.27–6.35)	6.3 (5.1–8.85)	0.049
CAP dB/m	293 (245.5–339)	212(178.75–226.5)	312 (273.5–344)	<0.001

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; LSM, liver stiffness measurement; CAP, controlled attenuation parameter; CRP, c-reactive protein

CAP and LSM Values According to Steatosis Degree and Fibrosis Stage. The distribution of patients in S0, S1, S2, and S3 steatosis degrees based on CAP score was 10 (5.5%), 30 (16.6%), 45 (24.9%), and 96 (53%), as shown in Figure 1.6, with a median CAP value for each degree of 212 (179–226), 245 (241–247), 273.5 (264–283), and 333 dB/m (312–357), respectively. According to steatosis grades, the median value of LSM was 5 kPa (4.27–6.35) in patients without steatosis (S0), 5.8 kPa (4.4–7) in mild steatosis (S1), 5.85 kPa (4.65–7.27) in moderate steatosis (S2), and 7.1 kPa (5.5–9.65) in severe steatosis (S3), with an increased LSM value among steatosis degrees ($p < 0.001$). From all patients included in the final analysis, 34 (18.8%) had advanced fibrosis (≥F3) with a median LSM value of 11.7 kPa (9.9–16.9). The LSM distribution according to meta-analysis of histological data in viral hepatitis fibrosis scale (METAVIR) was 73 (40.3%), 42 (23.2%), 32 (17.7%), 19 (10.5%), and 15 (8.3%) for F0, F1, F2, F3, and F4 grade, respectively, with a median for LSM score for each degree of 4.4 (3.8–5.1), 6.2 (5.8–6.2), 7.9 (7.45–8.5), 10.1 (9.7–11.05), and 17.1 kPa (14.55–21.85). Among fibrosis stages, the median CAP value was 270 (232–321), 290 (242.5–342.5), 312 (272.5–336.5), 321 (263–348), and 314 dB/m (278–373).

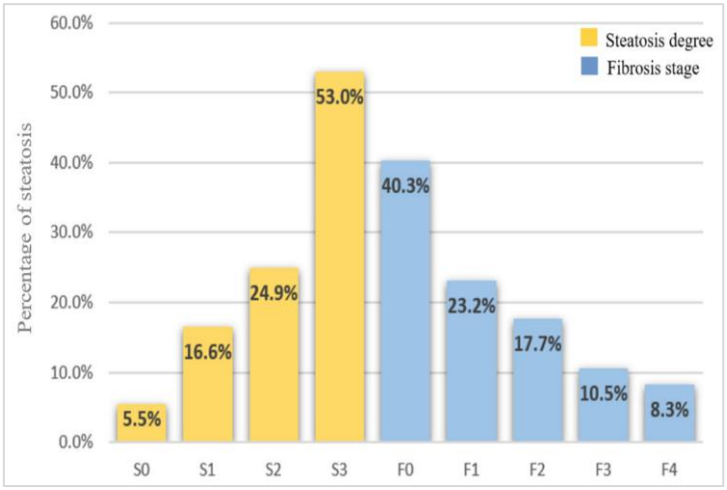

Fig. 1.6. Proportion of patients according to steatosis degrees and fibrosis stages.

Factors Associated with CAP and LSM. We performed a univariate linear regression analysis in order to identify risk factors associated with CAP and LSM, after which only those with a significant p value were included in multivariate regression analysis (Table 1.12).

Multivariate analysis showed that BMI ($\beta = 0.339$, $p < 0.001$), fasting plasma glucose ($\beta = 0.173$, $p = 0.046$), triglycerides ($\beta = 0.192$, $p = 0.043$), low-density lipoprotein cholesterol ($\beta = 0.155$, $p = 0.008$), and serum uric acid ($\beta = 0.192$, $p = 0.003$) were independent risk factors associated with CAP score in all patients. Age did not have a significant value in the multivariate analysis but remained strongly associated with the CAP value ($\beta = 0.144$, $p = 0.053$) in the univariate analysis. Furthermore, a lower platelet count and albumin level were also related to a higher LSM value, while serum uric acid ($\beta = 0.339$, $p = 0.028$) and alpha fetoproteine (AFP) ($\beta = 0.161$,$p = 0.033$) levels were independently associated with an increased LSM score. Overall, patients with increased LSM values were older (59.6 ± 7.49 vs. 57.4 ± 8.12 years) than those with decreased LSM values, although age was not associated with the LSM value in the univariate analysis ($\beta = 0.046$, $p = 0.537$).

Correlation between Surrogate Serum Fibrosis Markers, LSM, and CAP. For the overall cohort we found a significant correlation between LSM values and APRI ($r = 0.19$, $p = 0.020$), FIB-4 index ($r = 0.34$, $p < 0.001$), and NFS ($r = 0.30$ $p < 0.001$). Regarding hepatic steatosis expressed by CAP score, only NFS ($r = 0.25$, $p = 0.002$) maintained a positive, significant correlation, while APRI ($r = 0.11$, $p = 0.181$) and FIB-4 ($r = 0.07$, $p = 0.399$) did not.

Table 1.12. Factors associated with controlled attenuation parameter values and liver stiffness measurement using univariate and multivariate linear regression analysis

Variable	CAP				LSM			
	Univariate		Multivariate		Univariate		Multivariate	
	β	p	β	p	β	p	β	p
Gender	0.010	0.889			0.106	0.155		
Age, y	0.167	0.025	0.144	0.053	0.046	0.537		
BMI (kg/m^2)	0.542	<0.001	0.339	<0.001	0.244	0.002	0.094	0.299
Diabetes	0.233	<0.001	0.020	0.790	0.141	0.058		
Hypertension	0.382	<0.001	0.132	0.084	0.231	0.002	0.093	0.257
Platelet (G/L)	−0.008	0.913			−0.258	<0.001	−0.168	0.033
ALT (IU/L)	0.170	0.041	0.132	0.361	−0.013	0.874		
AST (IU/L)	0.172	0.039	0.115	0.889	0.038	0.650		
GGT (IU/L)	0.124	0.137			0.197	0.018	0.024	0.760
ALP (IU/L)	0.069	0.413			0.170	0.042	0.077	0.325
TB (mg/dL)	−0.086	0.304			−0.044	0.600		
Albumin (g/dL)	0.079	0.345			−0.295	<0.001	−0.276	<0.001
Creatinine (mg/dL)	0.044	0.600			0.057	0.494		
Urea (mg/dL)	0.150	0.044	−0.005	0.938	0.095	0.204		
Fasting plasma glucose (mg/dL)	0.160	0.031	0.173	0.046	0.045	0.551		
Ferritin (ng/mL)	0.092	0.218			0.015	0.845		
CRP (mg/dL)	−0.112	0.182			−0.014	0.867		
TC (mg/dL)	0.177	0.034	0.048	0.525	−0.102	0.225		
Triglycerides (mg/dL)	0.267	0.001	0.192	0.043	0.008	0.924		
LDL-c (mg/dL)	0.370	<0.001	0.155	0.008	0.127	0.131		
HDL-c (mg/dL)	−0.254	0.002	−0.008	0.913	−0.118	0.157		
Serum uric acid (mg/dL)	0.482	<0.001	0.244	0.003	0.339	<0.001	0.192	0.028
AFP (ng/mL)	0.075	0.369			0.211	0.011	0.161	0.033
LSM (kPa)	0.226	0.002	0.038	0.591				
CAP dB/m					0.226	0.002	0.060	0.540

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; LSM, liver stiffness measurement; CAP, controlled attenuation parameter; CRP, c-reactive protein, AFP, alpha-fetoprotein; FG, fasting plasma glucose; TB, total bilirubin

Discussion. NAFLD is the most common chronic liver disease of our century, reaching epidemic proportions, with approximately 25% of the global population affected (Younossi et al., 2016). NAFLD-related fibrosis is the strongest predictor of poor outcomes in these patients who are at high risk for developing cirrhosis and HCC (Bengtsson et al, 2019). Therefore, early detection of LF among NAFLD patients is mandatory for risk stratification and prevention of liver-related morbidity and mortality.

In our study, a high percentage of patients (94.5%) were diagnosed with hepatic steatosis (CAP >237 dB/m). Among them, 16.6% had S1, 24.9% had S2, and 53% had S3, proportions similar to those reported in another recently published study. Our patients with steatosis by CAP were older, more likely to associate hypertension and T2DM, with higher BMI, LSM, FIB-4 index, and NFS values than those without steatosis. In addition, upon multivariate analysis, we found that BMI ($\beta = 0.339$, $p < 0.001$), fasting plasma glucose ($\beta = 0.173$, $p = 0.046$), triglycerides ($\beta = 0.192$, $p = 0.043$), LDL-c ($\beta = 0.155$, $p = 0.008$), and serum uric acid ($\beta = 0.192$, $p = 0.003$) were independent risk factors associated with an increased CAP score.

Our results are similar to those reported by several studies. Thus, Kwok et al. evaluated 1918 patients using VCTE and CAP and reported that steatosis was associated with higher values of BMI, triglycerides, fasting plasma glucose, and alanin aminotransferase, and with a

low high-density lipoprotein cholesterol level, respectively (Kwok et al., 2015). A recent meta-analysis by Jarvis et al., including 22 unique studies, found that T2DM and a BMI >30 kg/m2 were linked with an increased risk of developing severe liver disease (Jarvis et al., 2020).

Most of our subjects had no LF (F0: 73, 40.3%) or presented only mild (F1: 42, 23.2%) fibrosis, while the proportion of patients with significant (F2: 32, 17.7%), advanced (F3: 19, 10.5%) fibrosis and cirrhosis (F4: 15, 8.3%) was lower.

It should be noted that the LSM value increased significantly in parallel with the degree of steatosis (S0 to S3, $p < 0.001$), with a strong correlation between LSM and serum fibrosis markers such as APRI ($r = 0.19$, $p = 0.020$), FIB-4 index ($r = 0.30$ $p < 0.001$), and NFS ($r = 0.30$ $p < 0.001$). These findings are in accordance with those revealed by several other studies comparing the accuracy of FibroScan in detecting fibrosis and steatosis stages among histologically confirmed NAFLD patients, which reported a prevalence between 10.1% and 28% for F3 stage, and 7.1–18.8% in patients with cirrhosis (De Leidighen et al., 2016; Eddowes et al., 2016; Tapper et al., 2016; Vuppalanchi et al., 2018; Oeda et al., 2019; Siddiqui et al., 2019).

On the other hand, we found by multivariate analysis a significant, negative association between platelet count ($\beta = -0.168$, $p = 0.033$), albumin level ($\beta = -0.276$, $p < 0.001$), and LSM value. These data are consistent with those from the current literature, showing that thrombocytopenia and hypoalbuminemia are negatively correlated with severe fibrosis and cirrhosis (Demir et al., 2014; Fallatah et al. 2016). We also found that serum uric acid ($\beta = 0.192$, $p = 0.028$) and AFP ($\beta = 0.161$, $p = 0.033$) are independent risk factors associated with an increased LSM value. Uric acid is related to the severity of liver damage as it promotes hepatic stellate cells activation and, subsequently, fibrosis development, while it is independently associated with HCC and NASH also in patients with NAFLD (Lanaspa et al., 2012; Jarunvongvanich et al., 2017; Rodriguez et al. 2019;). In a meta-analysis by Jaruvongvanich et al. that included five studies with biopsy-proved NAFLD patients, the authors found a significant correlation between hyperuricemia and NAFLD. Furthermore, subjects with elevated levels of serum uric acid had a high NAFLD activity score and a higher degree of histological liver damage (Jarunvongvanich et al., 2017).

The main limitation of our study was the absence of LB in the assessment of liver steatosis and fibrosis using only VCTE and CAP. In addition, we did not use different cut-off values for M and XL probes for fibrosis and steatosis evaluation. However, the prospective design of the study outweighs these shortcomings.

Conclusions. Our findings showed an important LF prevalence among patients with clinically suspected or previously diagnosed NAFLD by abdominal ultrasound examination. In addition, these results underline that the presence of metabolic syndrome components is a risk factor associated with both steatosis and fibrosis. Therefore, the assessment of LF and steatosis by VCTE and CAP should be carried out in all units with available facilities. Moreover, we believe that it is high time for a screening program to be developed for diagnosis and evaluation of NAFLD to reduce the risk of chronic liver disease progression.

Considering the rapidly changing landscape of the etiology of chronic liver diseases, with an optimistic prospect focusing on the elimination of viral hepatitis by 2030, NAFLD has begun to draw attention through its rising prevalence in the last decade. Our study underlines the importance of early diagnosis of NAFLD, which could lead to a decreased risk of developing severe fibrosis, thus lowering the mortality rates by liver-related events.

This direction of research is closely linked to the following one. In these two subchapters we have emphasized the importance and advantages of non-invasive investigations in patients with CLD. We cannot deny and eliminate methods of exploring invasiveness in medicine but it is preferable to use proven non-invasive methods when we have alternatives.

1.5. NON-INVASIVE EVALUATION OF LIVER FIBROSIS IN CHRONIC LIVER DISEASES

1.5.1. Introduction

Despite the multitude of newly discovered and validated non-invasive methods, liver biopsy (LB) still remains the gold standard method for assessing hepatic fibrosis and steatosis (Trifan and Stanciu, 2012). However, it is less and less used in practice due to several inconveniences such as intra- and interobserver variability, sampling errors, poor tolerability by the patient, high cost and especially because due to the rare risk of potentially life-threatening complications (Szymczak et al., 2012).

Our research team (under the mentoring of professor Stanciu) advocates for minimally invasive medicine which is why we were the first in Romania to support the replacement of LB in the evaluation of patients with viral hepatitis by non-invasive methods for determining the stage of LF.

Therefore, in the last years there has been a great number of methods developed for the noninvasive assessment (serological or imaging) for diagnostic, surveillance and treatment monitoring to determinate LF, that avoid the risks of invasive procedures (Trifan and Stanciu, 2012).

Transient elastography (TE) and VCTE are a highly accurate ultrasound-based technique, most widely used and accepted for the assessment of LF, with good sensitivity and specificity (Regev et al, 2002; Sasso et al, 2010). The major advantages of the noninvasive methods for assessing LF are mainly represented by quickness, painfulness, and easiness to perform; it is easily accepted by the patient, has a great repeatability and reproducibility (Vuppalanchi et al., 2018). These advantages allow us to assess the evolution/regression of LF at several timepoints in the evolution of CLD.

The noninvasive assessment of LF is mandatory in all patients diagnosed with CLD. The evaluation of LF is the most important criteria for the grading of CLD and the assessment of the rate of progression to liver cirrhosis.

1.5.2. Short-term changes of liver fibrosis in patients with HCV genotype 1b - related compensated cirrhosis after sustained virologic response

Background & Aim. TE is a highly accurate ultrasound - based technique, most widely used and accepted for the assessment of LF, with good sensitivity and specificity (Geng et al., 2016). The fibrosis index based on 4 factors (FIB-4) is one of the many noninvasive serological tests developed to detect LF in patients with chronic HCV infection. The FIB-4 index is a publicly available formula consisting of age, aspartate aminotransferase alanine aminotransferase and platelets, which has higher accuracy in predicting advanced fibrosis or cirrhosis when compared to LB (Turner et al., 2017).

This study aimed to assess the changes of fibrosis occurring after successful DAA treatment with noninvasive methods by analyzing LF measurements obtained with TE and FIB-4 index in patients with HCV genotype 1 b compensated cirrhosis, and to identify the risk factors associated with persistently elevated biomarker score and Fibroscan® related values after obtaining SVR.

Materials and methods. This study was a single-center, prospective observational cohort study which included 98 consecutive patients with HCV genotype 1b compensated cirrhosis who achieved SVR after DAA therapy with paritaprevir/ritonavir, ombitasvir and dasabuvir (PrOD) for 12 weeks in the Institute of Gastroenterology and Hepatology Iasi, from December 2016 to July 2017. For each patient, baseline demographics, clinical and laboratory data were recorded, and further data was collected during patient's visits at baseline, SVR and 12 months after the end of treatment. The diagnosis of cirrhosis was based on clinical, biochemical, imagistic features of liver cirrhosis, a liver stiffness ≥ 12.5 kPa measured with

Fibroscan® and a FIB-4 index ≥ 3.25. Exclusion criteria were co-infection with HIV or HVB, the presence of decompensated liver cirrhosis. Depending on the FIB-4 index value LF was categorized as nonsignificant, significant (≥F2 to ≤ F4) and cirrhosis (F4) as follows: <1.45, >1.45 and <3.25 and ≥ 3.25 respectively. A significant improvement of the serological test represents a decrease by ≥1-degree METAVIR score class.

LSM was performed by using TE (Fibroscan®, EchoSens, Paris, France) at baseline, SVR and 12 months after end of treatment (EOT). For a valid interpretation, at least 10 valid measurements were required with an interquartile range median (IQR/M) <30% and a successful rate > 60% (24). The median of LSM was ex-pressed in kilopascals (kPa) and cut-off values for fibrosis were as follows: (1) less than 7.1kPa for < F2, (2) from at least 7.1kPa to less than 9.5 kPa for ≥ F2, and (3) at least 9.5 kPa for ≥ F3, and at least 12.5 kPa for F4. More than 30% regression in LF, at SVR and 12 months after EOT, was considered a significant improvement.

The study was conducted in compliance with the Helsinki Declaration and was approved by the institutional review board. Written informed consent was obtained from all patients, at the start of the study.

Statistical analysis. Data were expressed as mean ± standard deviation, if normally distributed, and as median and interquartile-range (IQR), if not normally distributed. Friedman's test and Wilcoxon signed-rank test were carried out to detect longitudinal differences between noninvasive fibrosis assessment and clinical characteristics during follow-up-value value less than 0.005 was judged to be statistically significant. All the analyses were performed by using IBM SPSS 19.0 statistical software package (IBM corp., Armonk, NY, USA).

Results. We included 98 patients with HCV-related cirrhosis with DAAs-associated SVR. The baseline characteristics of all patients are summarized in table 1.13. The mean age of the studied patients was 60.64±9.56 years with a female predominance (65.3%) and a BMI of 28.16±4.28. Thirty-four (34.5%) patients were IFN-based regimens experimented, 18 (18.36%) had T2DM and 55 (59.12%) had hypertension. Liver stiffness at baseline was 21.30 (16.9-27.1) kPa.

Table 1.13. Patient characteristics

Variable	Baseline	SVR	12-month post-treatment
Age in years	60.64 ± 9.56	-	-
Female, *n* (%)	64 (65.3)	-	-
BMI (kg/m^2)	28.16 ± 4.28	-	-
HCV treatment - experienced, *n* (%)	64 (65.3)	-	-
Diabetes, *n* (%)	18 (18.4)	-	-
Hypertension, *n* (%)	55 (56.1)	-	-
Esophageal varices, *n* (%)	27 (27.6)	-	-
BMI (kg/m^2)	28.16 ± 4.28	28.00 ± 3.90	28.77 ± 3.85
Platelet (1000/µL)	155.74 ± 69.01	154.92 ± 62.47	171.96 ± 76.35
ALT (IU/L)	100.6 ± 75.96	39.62 ± 9.44	26.39 ± 16.31
AST (IU/L)	91.83 ± 61.88	34.14 ± 6.96	29.85 ± 14.35
GGT (IU/L)	87.46 ± 58.24	53.42 ± 9.33	43. 45 ± 49.77
Fasting sugar (mg/dL)	112.43 ± 31.73	99.60 ± 11.18	112.08 ± 29.01
Urea (mg/dL)	32.50 ± 9.94	39.20 ± 8.35	35. 81 ± 10.77
Direct bilirubin (mg/dL)	0.50 ± 0.30	0.35 ± 0.17	0.33 ± 0.17
INR	1.09 ± 0.10	1.08 ± 0.09	1.06 ± 0.07
Fibrinogen (mg/dL)	303.56 ± 66.36	311.09 ± 52.68	355.46 ± 42.37
Total cholesterol (mg/dL)	157.84 ± 36.84	174.09 ± 18.1	190.82 ± 38.55
LDL cholesterol (mg/dL)	97.09 ± 17.95	129.98 ± 25.88	125.40 ± 30.01

Variable	Baseline	SVR	12-month post-treatment
HDL cholesterol (mg/dL)	36.82 ± 5.68	41.38 ± 7.99	29.01 ± 6.67
Triglycerides (mg/dL)	106.79 ± 37.23	96.97 ± 22.63	121.13 ± 53
AFP (ng/mL)	15.82 ± 21.20	6.20 ± 5.89	5.46 ± 4.11
Albumin (g/dL)	4.12 ± 0.52	4.46 ± 0.39	4.50 ± 0.41
FIB-4	3.69 (2.19-6.02)	2.27 (1.55-3.26)	2.21 (1.51-3.28)
LSM (kPa)	21.30 (16.9-27.1)	16.45 (13.8-23.7)	15.65 (11.3-22.9)

Data are shown as mean ± SD, but LSM are expressed as median (interquartile range); BMI, body mass index; HCV, hepatitis C virus; ALT, alanine aminotransaminase; AST, aspartate aminotransaminase; GGT, gamma-glutamyl transferase; INR, international normalized ratio; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AFP, α-fetoprotein; LSM, liver stiffness measurement

Laboratory data at SVR and 12 months after end of treatment. Albumin, total cholesterol and low-density lipoprotein cholesterol levels significantly increased overall, while high-density lipoprotein cholesterol levels increased at SVR and recorded a significant reduction at 12 months after end of treatment. Significant differences were recorded between baseline vs. SVR, SVR vs. 12 months after end of treatment, and baseline vs. 12 months after end of treatment (Table 1.14). Triglycerides and INR levels showed no significant improvement.

Table 1.14. Patient P values for continuous clinical data

Variable	p-Value		
	Baseline *vs.* SVR	**SVR *vs.* 12-month post-treatment**	**Baseline *vs.* 12-month post-treatment**
BMI (kg/m^2)	0.400	< 0.001	< 0.001
Platelet (1000/μL)	0.918	0.020	< 0.001
ALT (IU/L)	< 0.001	< 0.001	< 0.001
AST (IU/L)	< 0.001	< 0.001	< 0.001
GGT (IU/L)	< 0.001	< 0.001	< 0.001
Fasting sugar (mg/dL)	0.003	0.001	0.608
Total bilirubin (mg/dL)	0.002	< 0.001	< 0.001
Direct bilirubin (mg/dL)	< 0.001	0.050	< 0.001
INR	0.008	0.084	0.001
Fibrinogen (mg/dL)	0.523	< 0.001	< 0.001
Total cholesterol (mg/dL)	< 0.001	0.001	< 0.001
LDL cholesterol (mg/dL)	< 0.001	0.265	< 0.001
HDL cholesterol (mg/dL)	< 0.001	< 0.001	< 0.001
Triglycerides (mg/dL)	0.077	0.001	0.351
AFP (ng/mL)	< 0.001	0.013	< 0.001
Albumin (g/dL)	< 0.001	0.104	< 0.001
FIB-4	< 0.001	< 0.001	< 0.001
LSM (kPa)	< 0.001	0.021	< 0.001

BMI, body mass index; HCV, hepatitis C virus; ALT, alanine aminotransaminase; AST, aspartate aminotransaminase; GGT, gamma-glutamyl transferase; INR, international normalized ratio; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AFP, α-fetoprotein; LSM, liver stiffness measurement;1 Wilcoxon signed-rank test was applied 2 Friedman's test was applied

Fibrosis evaluation assessed by TE and FIB-4 score

Overall baseline median (IQR) of LSM was 21.3 kPa (16.97-27.1) using Fibroscan®. At SVR, the median TE measurement was 16.45 kPa (13.8-23.7) and 15.65 kPa (11.3-22.9) at 12 months after end of treatment, with an overall median (IQR) regression of 4.5 (1.8-7.8) from baseline to SVR and 5.1 (2.5-8.65) from baseline to 12 months after EOT (p <0.001). Moreover, the LSM improvement is maintained from SVR to 12-month post-treatment (p

=0.021). LSM regression was found in 83 (84,6%) patients at 12 months after end of treatment evaluation. Subsequently, the primary outcome was obtained in 26 patients (26,53%) (p <0.001), which had more than 30% LSM decrease. The distribution of LSM over time is shown in Figure 1.6.

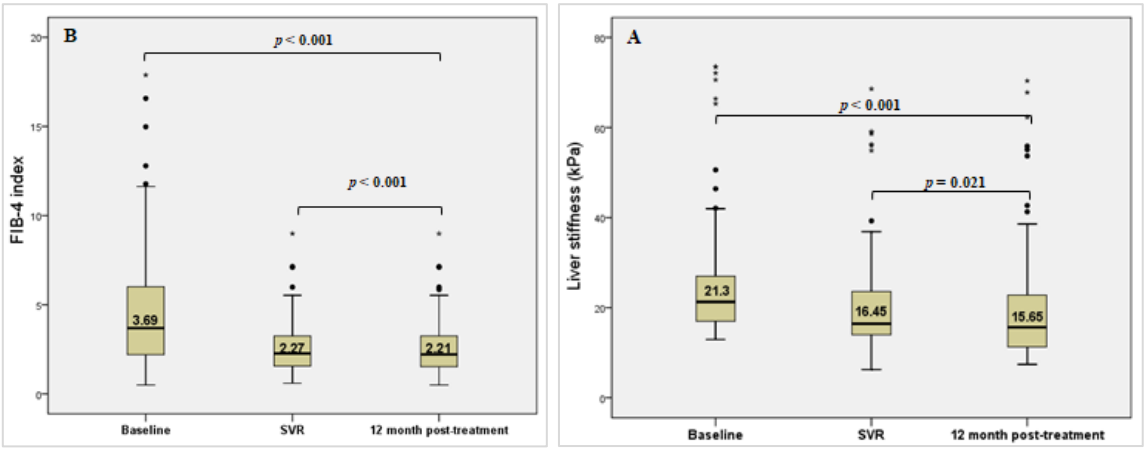

Fig. 1.6. Distribution of LSM (A) and FIB-4 index (B) over time. The lines through the boxes represent the median score. The upper and lower bottom of each box represents the 25th and 75th percentiles, representing the interquartile range (IQR). The rest of the outliner observations are plotted as dots beyond the whiskers. Each bottom error bar ends at 1.5*(IQR) < Q1, while each top error bar ends at 1.5*(IQR) > Q3.

The FIB-4 score significantly decreased at SVR and 12 months after end of treatment, similar to LSM values. At baseline, median (IQR) FIB-4 was 3.69 (2.19-6.02), while at SVR, the median (IQR) value reached 2.27 (1.55-3.26) with a decrease of 1.52 (0.37-2.75) (p <0.001). At 12 months after end of treatment, the median (IQR) was 2.21 (1.51-3.28), with a decrease of 1.52 (0.45-2.72) from baseline. The improvement of liver function expressed in FIB-4 score correlates with Fibroscan® METAVIR staging fibrosis. At 12 months after end of treatment, 73 (74.79%) patients had reached a down-grade of fibrosis stage from F4 (98) to F3 - F2 (50- significant fibrosis), < F2 (23)- nonsignificant fibrosis, while 25 remained at the same stage (F4).

In 13 patients there was a significant increase value of LSM median from 16.3 to 22.8 kPa while at 2 patients a stationary TE was reported. In this subgroup of patients, the median FIB-4 score was 2.43 from 4.3 initially, mean age of 65 years.

Discussion. Several previous studies conducted in the IFN era have shown a long-term improvement of LF in patients with SVR (Martinez et al., 2012; Kim et al., 2015). Given that DAAs have recently been introduced into the management of patients with chronic HCV infection, conclusive data on the course of long-term LF are still scarce.

This study seeks to enrich the current literature by prospectively evaluating long-term LF changes after obtaining SVR with DAA-based therapies. The key finding of our study consists of the long-term improvement in liver function demonstrated by a significant improvement of LSM and FIB-4 score. In our study, at one year of follow-up, 83 (84,6%) patients achieved a significant LSM decrease from baseline while 26 (26,53%) had a significant improvement of LSM (more than 30% regression in LF). A similar rate of improving LSM and FIB-4 score after SVR was reported by Mohammed et al., with a 46% reduction of LSM assessed by Fibroscan® and a >60% decrease of FIB-4 from baseline to one year (Mohammed et al., 2019). Another study which included patients treated with sofosbuvir-based DAA therapy, found a 31% reduction of liver stiffness after one year since EOT compared to baseline.

Even if LB remains the gold standard for estimating LF, in the last decade, noninvasive methods for evaluating the degree of LF are tempting in clinical practice. Their repeatability

and cost-effectiveness, besides patient convenience, represent sustainable arguments for abandoning LB in the long-term evaluation of HCV cirrhotic patients. Accordingly, a cross-sectional study by Hedenstierna et al. in which patients with SVR obtained with IFN-based regimens was followed-up through LSM for a period ranging from 5 to 10 years, concluded that the improvement of LF after viral clearance is significant in patients with pre-treatment advanced fibrosis or cirrhosis, but concerning long-term dynamics of fibrosis, regression becomes a slow process which at one point is stabilized (Hedenstierna et al., 2018). Therefore, the patients with more than 9.5kPa cut-off baseline values from their cohort had an improvement in LF. Only 21% of patients remained with more than 9.5 kPa at 10 years of follow-up. Also, the authors reported a progression of fibrosis in 5% of the patients, correlated with an age ≥ 55 years and a BMI ≥ 25 kg/m^2. In our study, we found an increase of TE score in 13 patients, while 2 patients had a stationary value. Of these subgroups, the mean values of age, BMI and FIB-4 index after DAA treatment were 65 years, 30.9 kg/m^2 and 2.43, respectively. We tend to conclude that age and BMI values after treatment are risk factors for fibrosis progression.

Our paper has some limitations. First, the small number of patients enrolled, secondly the lack of an LSM at EOT by FIB-4 score with a varied range of TE (±3 months). Thirdly, the short follow-up period represents a limitation because, for a true diminishing of LS, a longer follow-up period is required with a well-defined reevaluation-time. Fourthly, factors that are not HCV-related and might influence LSM such as alcohol consumption, steatogenic medication and lifestyle were not recorded.

Conclusion. TE was validated for assessing liver stiffness in patients with HCV-related liver cirrhosis, but long-term follow-up study based only on DAA based regimes treated patients are still limited. A rapid regression of fibrosis suggested by the improvement of LSM is secondary to the elimination of the hepatic inflammation after EOT. However, a dynamic assessment of LSM shows a tendency to settle on a constant value. In our patients, achieving SVR with DAA-based regimens was associated with significant improvement of liver stiffness measured by TE, and liver function monitored by FIB-4 index. A true regression of fibrosis must be constantly evaluated in dynamic, reason for which we intend to continue the TE follow-up of our cohort. Further studies are warranted to confirm these findings in other populations.

Summary of scientific contributions
Hereditary hemochromatosis - Our study was the first one that assessed the frequency of HFE gene mutations in Moldova. - HHC is often underdiagnosed - biological change in iron levels must be carefully evaluated.
Hepatitis C Virus Epidemiology - The ambitious goal of WHO to eliminate viral hepatitis as a public health problem by 2030 will require major efforts to increase the screening rates. - Our micro-elimination project carried out in a rural area is the first one in Romania; there are no similar projects in Europe carried out in rural areas and a few worldwide
Nonalcoholic fatty liver disease - The prevalence of NAFLD and LF in medical students is the only Romanian study that include this kind of population. - NAFLD has had a rising prevalence in recent years and standardized diagnostic methods are needed. - VCTE with CAP is a highly efficient method which could be easily used in screening programs.
Non invasive assessment of LF - Fibrosis regression is one of the major benefits of achieving SVR by DAAs - Non-invasive methods such as LSM are accurately demonstrating the changes of LF after the end of DAA treatment

Chapter 2

ADVANCED LIVER DISEASE – FROM BENCH TO BEDSIDE

Many of the habilitation theses have special research topics. I chose the patient as the main subject especially the patient with advanced liver disease because for over 25 years this has been my main concern both in terms of teaching and research but especially in my medical activity. I had patients whose evolution I have been following from the first moments of diagnosis with a chronic liver disease and unfortunately for many up to the stage of advanced liver disease with all its complications.

Scientific research in medicine is mandatory for clinical practice, for the practice of an evidence-based medicine with a primary impact on the patient's benefit (therapeutic, survival, etc.). All scientific results are implemented in medical practice and often real life data is a challenge for clinical trials and scientific research. It is obvious that the aphorism 'bench to beside to bench' must promote medical activity.

2.1. INTRODUCTION. CHRONIC LIVER DISEASE - OLD TOPIC ALWAYS CURRENT

Chronic liver disease has been for many years a global health problem. Liver cirrhosis (LC) - the end stage of all chronic liver diseases regardless of etiology - is one of the leading causes of morbidity/mortality and health problem worldwide (Zatonski et al., 2010; Blachier et al., 2013; Mokdad et al., 2014; Global Hepatitis Report 2017).

Over the last 30 years mortality has dropped in most of chronic diseases (cardiovascular, metabolic, pulmonary and even cancer). Unfortunately, CLD has an ascending mortality trend (Global Hepatitis Report 2017).

3.5% of all deaths worldwide are due to liver cirrhosis and hepatocellular carcinoma. LC is currently the 11th most common cause of death globally (Practice guideline by EASL and American Association for the Study of Liver Diseases (AASLD), 2014; Global Hepatitis Report, 2017; Asrani et al., 2019; Sepanlou et al., 2020; Cheemerla and Balakrishnan, 2021).

The latest hepahealth report (Pimpin et al., 2018) showed that the age-adjusted prevalence for the latest year in 35 countries for males and females ranged from 447 (Iceland) to 1,100 (Romania). The same report showed that Romania has the highest death rates (36 per 100,000) compared to other countries (the lowest rates, below 10 per 100,000 are reported in Iceland and Norway (Pimpin et al., 2018).

The last Baveno Consensus has redefined compensated/decompensated LC - compensated cirrhosis is defined by the absence of present or past complications of cirrhosis (De Franchis et al., 2021). So, any previous decompensation of liver disease means decompensated cirrhosis, even if at some point clinical, biological data show compensation for the disease. The same consensus nuanced the criteria for decompensating liver cirrhosis; the events that define decompensation in a compensated cirrhosis are overt ascites (or pleural effusion with serum/pleural effusion albumin gradient > 1.1 g/dl), overt hepatic encephalopathy (West Haven grading> II) and variceal bleeding. There are inconsistent data on whether minimal ascites (imaging procedures detected only), covert encephalopathy or occult bleeding due to portal hypertensive gastropathy can be considered as cirrhosis decompensation. The same Baveno Consensus points out that isolated jaundice (except for cholestatic diseases) is very rarely the first sign of decompensation of compensated cirrhosis and in these patients we must exclude first of all superimposed liver injury or ACLF.

Advances in the treatment of chronic viral hepatitis in recent years (cure of hepatitis C virus infection, vaccination for hepatitis B and viral suppression in patients with hepatitis B virus infection) have led us to the hope of reducing morbidity and mortality in patients with chronic liver disease (Moon et al., 2020; Cheemerla and Balakrishnan, 2021).

Moreover, the WHO aims to eliminate viral hepatitis as a major public health by 2030. This goal involves a 90% reduction in new cases of HBV and HCV infections by 2030 and a 65% mortality reduction by 2030 (WHO Combating hepatitis B and C to reach elimination by 2030. 2016. Available at: www.who.int/hepatitis/publications/hep-elimination-by-2030-brief/en/). Treating HCV with DAAs is mandatory both for reducing current infections and preventing new ones. In Romania, access to treatment is easy but unfortunately worldwide less than 10% of people infected with HCV have been treated and cured since 2014 when DAAs is the standard of treatment for HCV. The treatment cost is very high this is why unfortunately its access in developing countries is still restricted or non-existent (EASL Recommendations on Treatment of Hepatitis C, 2018).

The change in the etiology of advanced liver disease has also altered the indications for liver transplantation (LT) in these patients. In 2020 Wong and Signal showed that NASH and ALD have become the most common etiologies of liver disease among patients without HCC, on LT list and NASH is becoming a leading indication in patients with HCC (Wong and Signal, 2020).

Unfortunately, the reduced morbidity and mortality in patients with chronic liver disease does not seem to be possible as the burden of chronic liver disease will remain a major health problem in the coming years while the incidence of fatty liver disease continues to rise (Mahli and Hellerbrand, 2016; Ott et al., 2017; Ford et al., 2017; Estes et al., 2018).

In addition, from our point of view in our geographical area regarding the main alcoholic etiology, liver cirrhosis will continue to be a health problem, a pathology with high need for medical care, high costs, with an increased number of hospitalizations and readmissions. All our studies and papers presented at national conferences have shown that the main etiology of liver cirrhosis in our patients is the alcoholic one.

Our most recent NASH study showed that 94.5% of patients referred by general practitioners with clinically suspected or US diagnosed NAFLD, were diagnosed with hepatic steatosis (CAP ≥237 dB/m) (Zenovia et al., 2021).

Despite all the therapeutic progress in hepatology so far, the bench to bedside and back result is far from satisfactory. As long as we cannot effectively prevent/ intervene on ALD and NASH it is unlikely that we will testify a significant reduction in patients with advanced liver disease in the future.

In conclusion, we will still have patients with liver cirrhosis with all its complications that we have to treat and will have to keep alive until liver transplantation.

Given these aspects, I will refer to the main complications of CLD, emphasizing our main contributions in the field.

The scientific expertise in this field is supported by the scientific and editorial activity:

Articles published in ISI indexed journals		**WOS citations**
1.	Muzica CM, Stanciu C, Huiban L, Singeap AM, Sfarti C, Zenovia S, **Cojocariu C,** Trifan A. Hepatocellular carcinoma after direct-acting antiviral HCV therapy: A debate near the end. *World J Gastroenterol* 2021 **IF – 5.22**	13
2.	Sfarti C, Ciobica A, Balmus IM, Trifan A, Petrea O, **Cojocariu C***, et al. Systemic Oxidative Stress Markers in Cirrhotic Patients with Hepatic Encephalopathy: Possible Connections with Systemic Ammoniemia. *Medicina (Kaunas)*. 2020;56(4):196. doi:10.3390/medicina56040196. **IF - 1.5**	2
3.	Trifan A, Stoica O, Stanciu C, **Cojocariu C**, Singeap AM, Girleanu I, Miftode E. *Clostridium difficile* infection in patients with liver disease: a review. *Eur J Clin Microbiol Infect Dis.* 2015 Dec;34(12):2313-24. **IF 2.72**	24

	Articles published in ISI indexed journals	WOS citations
4.	Stefan Chiriac, Carol Stanciu, **Camelia Cojocariu***, Ana-Maria Singeap, Catalin Sfarti, Tudor Cuciureanu, Irina Girleanu, Razvan Alexandru Igna, Anca Trifan. Role of ammonia in predicting the outcome of patients with acute on-chronic liver failure. *World J Clin Cases 2021* January 26; 9(3): 552-564 **IF – 1.013**	
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6.	Irimia R, Carol Stanciu, **Camelia Cojocariu**, Cătălin Sfarti, Anca Trifan. Oral Glutamine Challenge Improves the Performance of Psychometric Tests for the Diagnosis of Minimal Hepatic Encephalopathy in Patients with Liver Cirrhosis. *J Gastrointestin Liver Dis*, September 2013 Vol. 22 No 3: 277-281 **IF – 1.849**	7
7.	Stoica OC, Stanciu C, **Cojocariu C**, Miftode E, Boiculese L, Trifan A, Girleanu I. *Clostridium difficile* Infection in Hospitalized Cirrhotic Patients with Hepatic Encephalopathy. *J Gastrointestin Liver Dis*. 2015 Dec;24(4):423-8. doi: 10.15403/jgld.2014.1121.244.csd. PMID:26697567. **IF 1.837**	
8.	Girleanu I**,** Anca Trifan, Laura Huiban, Cristina Muzica, Roxana Nemteanu, Andreea Teodorescu, Ana Maria Singeap, **Camelia Cojocariu**, Stefan Chiriac, Oana Petrea, Sebastian Zenovia, Robert Nastasa, Tudor Cuciureanu, Carol Stanciu. The risk of *Clostridioides difficile* infection in cirrhotic patients receiving Norfloxacin for secondary profilaxis of spontaneous bacterial peritonitis- A real life cohort. *Medicina* (Kaunas) 2021; 57(9):964. IF **-2.430**	
9.	Girleanu I, Stanciu C, **Cojocariu C**, Boiculese L, Singeap A, Trifan A. Natural course of nonmalignant partial portal vein thrombosis in cirrhotic patients. *Saudi J Gastroenterol* 2014;20:288-92. **IF 1.00**	19
10.	Muzica CM, Stanciu C, Cijevschi-Prelipcean C, Girleanu I, Huiban L, **Cojocariu C**, et al. Long-term Risk of Hepatocellular Carcinoma Following Direct-Acting Antiviral Therapy in Compensated Liver Cirrhosis Induced by Hepatitis C Virus Infection. *Hepat Mon*. 2021;21(6):e115910. doi: 10.5812/hepatmon.115910. **IF – 0.66**	
	Articles published *in extenso* in ISI proceedings journals	
	Cojocariu Camelia, Stanciu Carol, Singeap Ana Maria, Sfarti Catalin, Negru Dragoș, Trifan Anca. Influencing the Consequences of Portal Hypertension. Endoscopic Treatment, Early Transjugular Intrahepatic Portosystemic Shunt. *Proceedings of the XXXVI National Congress of Gastroenterology, Hepatology and Digestive Endoscopy*, Cluj Napoca, jun 2016, ISBN – 978-88-95922-72-0: 131	
	Irina Girleanu**,** Carol Stanciu, Dan-Stefan Teodorescu, Oana Cristina Petrea, **Camelia Cojocariu**, Ana Maria Singeap, Catalin Sfarti, Andreea Teodorescu, Roxana Nemteanu, Stefan Chiriac, Laura Huiban, Cristina Muzica, Tudor Cuciureanu, Anca Trifan. Predictors of in-hospital mortality in a cohort of elderly cirrhotic patients with variceal bleeding. *Proceedings of the Central European Gastroenterology Meeting (CEURGEM)* 2019; 66-70	
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	Articles published *in extenso* in BDI indexed journals	
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2.	**Camelia Cojocariu**, Anca Trifan, Sidonia Bucătaru, Alina Andriucă, C. Stanciu. Hemoragia digestivă superioară de cauză variceală. În C. Stanciu *Curs intensiv de boli digestive*. Ed "Gr T Popa", Iasi, 2013: 179-191. ISBN 978-606-544-154-5	
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Awarded papers		
	AASLD Poster of distinction. Chiriac Ș, Stanciu C, Sîngeap AM, Sfarti C, **Cojocariu C,** Gîrleanu I, Cuciureanu T, Stoica O, Huiban L, Muzica CM, Livadariu RM, Trifan A. Relative adrenal insufficiency in the setting of acute on chronic liver failure – an organ dysfunction that could indicate futility. Digestive Disease Week, 2-5 iunie 2018, Washington	
	Italian Society for the Study of Ecstasy and Thrombosis - ***Young Researcher Award.*** I. Girleanu, A. Trifan, **C. Cojocariu,** M. Dimache, A. Singeap, O. Stoica, C. Stanciu. Natural course of de novo partial portal vein thrombosis in patients with cirrhosis 5th International Congress on Coagulopathy in Liver Disease, Padua, 27-28 September 2013	
Total WOS citations		**98**
Cumulative impact factor/direction		**20.066**

2.2. GASTROINTESTINAL BLEEDING IN PATIENTS WITH LIVER CIRRHOSIS

2.2.1. Introduction

Gastrointestinal bleeding is one of the common complications in patients with LC. The causes are numerous but certainly the most common is variceal bleeding (VB). VB is one of the major complications of LC, the most dramatic in gastroenterology and one of the main causes of death in cirrhotic patients. Mortality and the risk of variceal rebleeding are high in the first 6 weeks following the first episode of VB.

Despite the progresses and standardization of the pharmacological and endoscopy therapy in the last 3 decades mortality rates remain markedly high (17-20%) especially in patients with advanced liver disease (Chalasani et al., 2003; D´Amico and De Franchis, 2003; Reverter al. 2014). In a recent study, Matei et al, showed that in Romania the severity of cirrhosis is an important prognostic factor for failure of control bleeding and 6 weeks mortality (Matei et al., 2022). They showed that identifying the factors associated with rebleeding and early mortality could be helpful in selecting patients for the most efficient therapy and the need of more than just conventional therapy. The evaluation of portal venous pressure and even emergency vascular interventions could improve the management of these patients.

In 2007 we presented on National Congress of Gastroenterology and Hepatology the first results of sandostatin administration in variceal bleeding in Romania. I must emphasize that at that time we were the only health unit in the country were pharmacologically treatment for variceal hemorrhages was applied according to specialized guidelines. More, not long after the meeting we have implemented in Romania under the guidance of Prof. Carol Stanciu the standardized treatment of acute variceal bleeding in cirrhotic patients. Between 1997 and 2006, the number of patients with variceal bleeding hospitalized in IGH increased steadily; in 2006 the number of patients was double compared to 1997. Analyzing the death rate while not using vasoactive medication compared to the period when routine administration of sandostatin was implemented in current practice we found a progressive reduction in mortality; from a death rate of 39.3% in 1997 to 18.2% in 2006 (Cojocariu et al., 2007). A few years later we compared the efficacy of octreotide vs terlipressin in the treatment of variceal bleeding (Girleanu et al., 2010). Mortality during admission was similar in both groups (14.63% terlipressin *vs*. octreotide 14.10%, p = 0.512) and even lower that in 2007.

The clinical expertise in this field is supported by the activity in the acute therapy unit and provided medical assistance for major gastroenterological emergencies. Moreover, I consider that gastrointestinal bleeding in a cirrhotic patient is the most dramatic emergency in gastroenterology.

2.2.2. Predictors of In-hospital Mortality in a Cohort of Elderly Cirrhotic Patients with Variceal Bleeding

Background & aim. VB a frequent complication of liver cirrhosis has high rates of morbidity and mortality, especially in elderly population. Early identification and management of the factors predicting in-hospital mortality might decrease mortality. The aim of this study is to evaluate the predictive factors for in-hospital mortality in elderly cirrhotic patients with variceal bleeding.

Material and methods. Cirrhotic patients aged ≥65 years presented with VB from January 2017 to December 2017 in our hospital, were included in the study. The patients with a past history of endoscopic treatment, variceal hemorrhage, Child-Pugh classification of C, extra- hepatic metastasis of HCC and/or portal vein tumor thrombosis of HCC were excluded. The clinical data were investigated retrospectively.

The data was collected from patients' personal files, where they have signed an informed consent regarding the study, they are included in. The diagnosis of liver cirrhosis was established based on clinical manifestations and biological, endoscopic, and ultrasound changes suggestive for advanced liver disease and portal hypertension. Liver function was quantified by Child-Pugh (Cholongitas et al., 2005) and Model of End-Stage Liver Disease (MELD) scores (Durand and Valla, 2005). The presence of ascites was assessed by clinical examination and abdominal ultrasound. VB was confirmed by upper endoscopy examination performed at the admission or in the first 12 hours after admission. VB was defined as the presence of active bleeding, an adherent clot, nipple sign or cherry spots on the variceal walls. Patients were followed up to 30 days, discharge from hospital or death (whichever came first).

Statistical analysis was performed using SPSS Software Version 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean ± standard deviation and categorical variables as frequency and percentage. Student's t test was used to compare normally distributed continuous variables and the Mann–Whitney U test for variables without normal distribution. The Chi-square test and Fisher approximation method were used to compare categorical variables. Logistic regression models were used to identify clinical variables associated with mortality. Univariate analysis was performed for each recorded variable. Variables with P-value <0.01 in univariate analysis were included in multivariate analysis. For adjusted effect we used the logistic regression. The level of statistical significance was defined as P-value <0.05.

Results. In this study we included 75 elderly cirrhotic patients, mean age 70.29±4.8 years (range 65-83 years), most of them females (60.0%). The first three etiologies of LC were alcoholic (62.7%), viral C infection (22.7%) and viral B infection (14.6%).

The majority of the patients had mean Child-Pugh score 9.93±3.17, with a mean MELD score of 15.56±7.7. Of the 75 patients evaluated, 48 (64.0%) had other comorbidities as: diabetes mellitus, arterial hypertension, atrial fibrillation, chronic kidney disease or neurological disease. Eleven (14.6%) patients had an anticoagulant treatment for stroke prevention. Fifty-eight patients (77.3%) were previously diagnosed with esophageal varices, and only 22.1% of those who needed primary VB received beta-blockers. In the majority of cases the upper digestive endoscopy was performed at the admission (74.7%), the main source of bleeding were the esophageal varices- 67 patients (89.3%), and only 8 patients (10.7%) bled from gastric varices.

Variceal active bleeding was confirmed in 27 patients (36.0%). Variceal banding and terlipressin was the treatment in 22 patients (29.3%), the rest of the patients receiving only the vasoactive treatment. Blackmore probe was inserted in 21 patients (28.0%) with rebleeding after banding or they had a hemorrhagic shock. Twenty patients (26.7%) died during hospitalization, the causes of death being: hemorrhagic shock, hepatic coma or multiple organ failure.

Table 2.I. Univariate and multivariate regression analysis of risk factors associated with death in cirrhotic patients with variceal bleeding

Parameter	Univariate analysis			Multivariate analysis		
	OR	95%CI	p-Value	OR	95%CI	p-Value
Age > 75 years	1.937	1.328 – 3.231	0.009	1.591	1.029 – 1.866	0.011
Hemodynamic instability at presentation	8.351	2.265 – 16.549	0.005	11.512	1.078 – 133.41	0.043
Comorbidities	2.888	1.929 – 7.133	0.037	4.918	1.954 – 12.637	0.001
Failure to control bleeding	2.494	1.934 – 7.133	0.003	2.031	1.388 – 16.227	0.029
Anticoagulant treatment	1.294	0.128 – 4.668	0.554	1.348	0.128 – 15.102	0.109
Creatinine > 1.2 mg/dl	1.373	0.694 – 2.718	0.411	-	-	-
Ascites	1.147	0.951 – 1.383	0.347	-	-	-

The multivariate analysis demonstrated that increasing age (OR 1.59, CI 1.029-1.866, P=0.011), hemodynamic instability at presentation (OR 11.51, CI 1.078-133.41, P=0.043), comorbidities (especially diabetes mellitus and chronic kidney disease) (OR 4.918, CI 1.954-12.637, P=0.001), and failure to control bleeding (OR 2.031, CI 1.388-16.227, P=0.029) were independent risk factors, significantly associated with in-hospital mortality among cirrhotic elderly patients presented with VB (Table 2.I).

Discussions. In our study we showed that VB has a higher rate of mortality in elderly cirrhotic patients despite the maximal treatment, and the rate of beta-blockers are less indicated in this special category of cirrhotic. The in-hospital mortality rate in general population range between 8%- 14.2% (Bambha et al., 2008). The comorbidities, especially diabetes mellitus and chronic kidney failure, failure to control bleeding, hemodynamic instability at admission, and age over 75 years old were recognized as mortality risk factors. Mortality was related to the severity of bleeding episode as expressed by hemodynamic instability at admission and presence of comorbidities. Our results are in line with previously published data (Han et al., 2014). The incidence of gastrointestinal bleeding increases with aging and accounts for 1% of hospital admissions in people aged 80 years and over (Kaplan et al., 2001; Tariq et al., 2007). Aging per se is a risk factor for gastrointestinal bleeding, regardless of other predisposing factors, as it has been recently demonstrated in a large prospective study on people who were not taking antiplatelet agents (Selak et al., 2018). The in-hospital mortality rate in our study was higher than previously reported (Wang et al., 2012) may be in relation with the number of the comorbidities and the association of failure to control bleeding. The mortality rate in elderly with non-variceal upper hemorrhages was reported up to 10% lower that the mortality rate in cirrhotic patient with VB (Tariq et al., 2007). Considering LC as a systemic disease, especially in the advanced stages, it could represent a risk factor for mortality in elderly. Acute VB is associated with a poor prognosis in old cirrhotic patients, determining further decompensation of the disease. Mortality was related to the failure to control bleeding and in-hospital complication. In hospital rebleeding and complications played a highly significant role for stratification of patients with high mortality. Both occurred after admission which indicates that prognosis may change from day to day. This necessitates intensive monitoring of elderly cirrhotic patients with acute VB during hospitalization and further improvement to control bleeding might still improve the prognosis of these patients. The rate of primary prophylaxis with beta-blockers was lower in our study group, many patients having contraindication for non-selective betablockers as: atrio-ventricular block, peripheral arterial disease or obstructive pulmonary disease (Motivala et al., 2016).

Conclusion. In-hospital mortality rate was 26.7% and the predictive factors of in-hospital mortality for the cirrhotic elderly patients were increasing age, hemodynamic instability at presentation, comorbidities and failure to control bleeding. These patients may require care in more specialized units during the bleeding episode, intensive monitoring, energetic resuscitation, early endoscopy, various options to control bleeding and aggressive follow-up in the intensive care units.

Our study has some strengths and also several limitations. Strength - it is one of the fewest studies designed specifically to investigate the mortality rate from VB in elderly cirrhotic patients. Limitations it is a retrospective study, with relatively small sample size.

With the increase in life expectancy, we have more and more elderly patients with LC. Primary and secondary prevention of VB can be a challenge in these patients because many of them have contraindications for beta-blockers.

Another topic of study was the incidence of VB in patients with SVR treated with DAAs. DAAs have radically changed the management of chronic HCV infection over the past

years through high effectiveness and safeness, with a major impact on the prognosis and burden of the disease. Unfortunately, the risk of VB remains high in cirrhotic patients with DAA's treatment. They may have gastrointestinal bleeding during antiviral therapy, which sometimes poses particular problems with monitoring and treatment. Secondly, despite SVR in HCV patients on the short time the liver fibrosis and portal hypertension remain and is associated with the risk of digestive bleeding as in any cirrhotic patient.

2.2.3. Bleeding events in patients with HCV - related liver cirrhosis treated with direct acting antivirals

Background & aim. Sustained virologic response is now achieved in >90% of the DAAs treated patients and is associated with improvements in liver function, fibrosis and overall survival assessed by the Child-Pugh and MELD score. Portal hypertension is also expected to improve with virological response, along with the improvements in liver inflammation and liver fibrosis (Nahon Pet et al., 2017). The knowledge of the effects of HCV elimination on clinically significant outcomes like portal hypertension and its complications is thus of unremarkable importance since it can influence management after SVR.

There are no conclusive data regarding the effect on clotting disorders due to liver cirrhosis (Tripodi et al., 2017).

In our study, we aimed to assess the incidence and risk factors of bleeding events in cirrhotic patients with SVR treated with DAAs.

Materials and methods. This retrospective study included patients with HCV-related liver cirrhosis (diagnosis established by abdominal ultrasound -Philips HD 11xe ultrasound system- and non-invasive techniques for assessing liver fibrosis (Fibroscan and Fibromax) which fulfilled the criteria for antiviral therapy; the study was conducted in a tertiary gastroenterology referral center from North-Eastern Romania, between January 1st, 2016 and January 1st 2019. The inclusion and exclusion criteria where according to our National Protocol. The regimens used were: Paritaprevir/Ritonavir, Ombitasvir and Dasabuvir (PrOD) ± Ribavirin (RBV) in patients with HCV-related compensated liver cirrhosis Child Pugh A5 and Ledipasvir/Sofosbuvir (LED/SOF)+RBV for 12/24weeks, in patients with compensated/decompensated LC.

The study protocol was approved by the Ethical Research Committee of "Grigore T. Popa" University of Medicine and Pharmacy Iasi and by the Ethical Committee of the "Sf. Spiridon" County Clinical Emergency Hospital. All patients signed an informed consent.

Blood tests were performed at baseline, at12/24 weeks after end of treatment and during the presentation for digestive bleeding. All patients with presumption of digestive bleeding (hematemesis or melena) were evaluated by upper digestive endoscopy performed in emergency and blood samples.

Statistical analysis. Data collected was statistically analyzed using SPSS 20.0 (Chicago, IL, SUA). tests were two-tailed with p-value <0.05 was considered statistically significant. The quantitative variables were compared using the t-Student test, and the qualitative parameters were evaluated by the chi-square test.

Results. The study included 874 HCV-infected cirrhotic patients treated with PrOD or LED/SOF± RBV with documented SVR, mean age58,7 ± 6,2 years, predominantly female (58%). 443 (50.68%) patients were PrOD treated and 431 (49.31%) of them were treated with LED/SOF ± RBV. 721 (82.49%) patients had compensated LC and 153 (17.50%) had decompensated LC and Child-Pugh score at baseline was 8.64 ± 1 in the LED/SOF ± RBV group *vs*. 5.06±0.24 points (Table 2.II).

Table 2.II. Demographic characteristics at baseline

Demographic characteristics at baseline	All (n = 874)
Females, n (%)	507 (58%)
Age (mean ± SD)	58.7 ± 6.2
Treatment Regimen	
PrOD, n (%)	443 (50.68%)
LED / SOF + RBV, n (%)	431 (49.31%)
CHILD – PUGH Score	
PrOD (mean ± SD)	5.06 ± 0.24
LED / SOF + RBV, (mean ± SD)	8.64 ± 1
Stage of liver disease	
Compensated LC	721 (82.49%)
Decompensated LC	153 (17.50%)

Mean period between SVR and the occurrence of bleeding events was 230±121 days. Bleeding complications after SVR were reported in 16 (1.83%) patients: 9 (56.25%) with variceal hemorrhage and 7 (43.75%) with non-variceal hemorrhage (portal hypertensive gastropathy, gastric ulcer, duodenal ulcer, or Mallory-Weiss syndrome) (Figure 2.1).

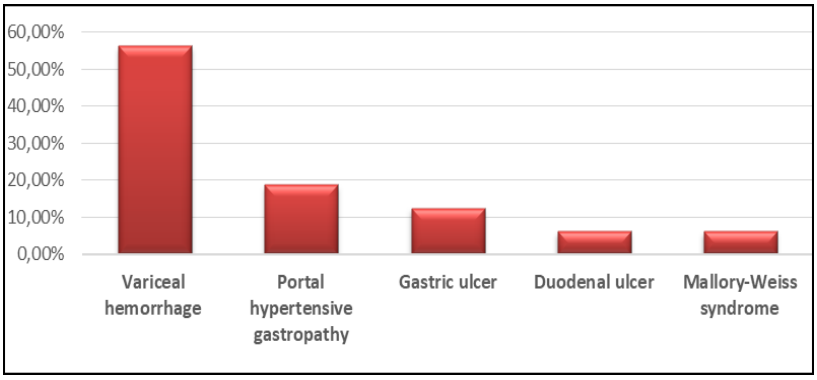


Fig. 2.1. Bleeding events after SVR

Four patients had previous episodes of variceal hemorrhage before initiating DAAs therapy.

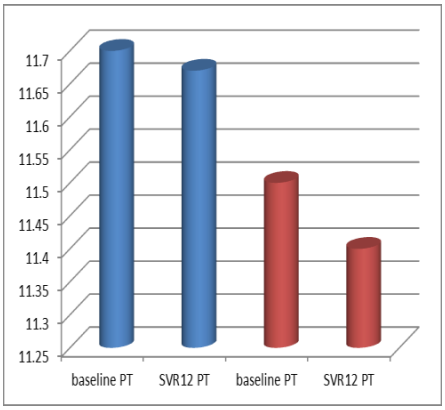


Fig. 2.2. Prothrombin serum levels at baseline and SVR12

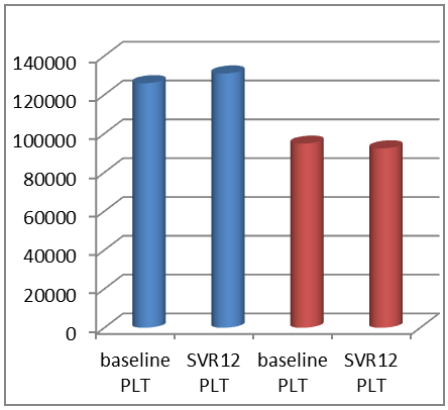


Fig. 2.3. Platelets count at baseline and SVR

There was no significant change in prothrombin serum levels in both groups: baseline values in patients treated with PrOD was 11.67±0.91 versus 11.70±0.83at SVR, p=0.993, respectively 11.5±0.84 sec at baseline versus 11.4±0.68 at SVR, p=0.715 in patients treated with LED/SOF+ RBV (Figure 2.2).

Platelet count had no significant changes at baseline versus SVR in both groups (126 000 (101 500-162 000) vs. 131 000 (101000-165 000), p= 0.818 in patients treated with PrOD, respectively 94857.14±32 vs. 92428.57±35, p= 0.853, in patients treated with LED/SOF+RBV) (Figure 2.3).

Discussions. The last few years brought an important change in the therapeutic management of chronic HCV infection. DAAs are highly effective and safe and are changing the prognosis and burden of the disease. SVR is now achieved in more than 90% of cases and is associated with improvements in liver function, fibrosis and overall survival (Goldberg et al., 2017).

Although thrombotic events may occur in the natural course of liver cirrhosis, bleeding events are more frequent in clinical practice. There are very few studies regarding the coagulation status of cirrhotic patients with SVR treated with DAAs (Tripodi et al., 2017).

Our study showed an incidence of 1.83% of bleeding events in patients with HCV related liver cirrhosis treated with DAAs.

There was a slight improvement of platelet count after SVR in patients treated with PrOD, but not significantly statistic. We didn't find any improvement in platelet count at SVR compared to baseline in patients treated with LED/SOF+RBV, but on the contrary we identified a slight decrease in platelets that can be explained by splenic sequestration and exaggerated destruction, representing a characteristic status in patients with advanced liver disease.

Libanio and Marinho evaluated the changes in hepatic venous pressure gradient (HVPG) and liver stiffness in 60 cirrhotic patients (84% Child A) treated with various combinations of DAAs and they showed that SVR led to a reduction in HVPG in 80% of the patients (Libanio and Marinho, 2017).

According to Calvaruso et al although portal hypertension may improved in the majority of HCV patients after SVR varices may continue to progress despite successful hepatitis C treatment (Calvaruso et al., 2018).

Unfortunately, once patients develop cirrhosis with severe portal hypertension (HVPG ≥ 10 mmHg), regardless of SVR they remain at risk for hepatic decompensation within the first 5 years after treatment (Sabela et al., 2015).

Due to the novelty of DAAs therapies there are only few studies assessing it's effects on portal hypertension and clinical decompensation.

Conclusions. Bleeding events in patients with HCV- related liver cirrhosis treated with DAAs are not influenced by the variations of coagulation parameters, rather correspond to the hemodynamic changes induced by the status of advanced liver disease. It is not clear if the mechanism of DAAs can influence the development of bleeding events in these patients or are part of the natural course of advanced liver disease.

Despite SVR in HCV patients on the short time the liver fibrosis and portal hypertension remain and is associated with the risk of digestive bleeding as in any cirrhotic patient.

The bleeding events in cirrhotic patients may be caused by portal hypertension (esophageal, gastric, ectopic varices, portal gastropathy) or in the advanced stages of the disease by the status of hypocoagulability that could be associated with the appearance of cutaneous mucosal or intracranial bleeding.

VB remains one of the most severe and dramatic complications of liver cirrhosis being the most significant complication of portal hypertension. It is associated with a high early mortality (20% at 6 week).

Another research topic, imposed by clinical activity, was and remains hepatic encephalopathy (HE); EH is an unpredictable complication that often requires acute therapy

assistance. The professional activity in this pathology is proved by the large number of cirrhotic patients managed for HE. Consistent with scientific research and provoked by data from the literature we also studied some pathogenic aspects of HE.

2.3. HEPATIC ENCEPHALOPATHY

2.3.1. Introduction

HE is the second most common complication of liver cirrhosis (after ascites) with a significant negative impact on the patient survival. Nearly 30%-45% of patients with liver cirrhosis have at least one episode of overt HE and the annual risk of developing HE in cirrhotic patients is 20% (Poordad, 2007); it is recognized that 30% of deaths in patients with end-stage liver disease are in those with severe encephalopathy/hepatic coma (Ferenci, 1995).

HE is a major reason for hospitalization and early/later readmission of cirrhotic patients and the economic burden is substantial. Prophylaxis and treatment of complications of liver cirrhosis is an important goal in any cirrhotic patient. Volk et al showed that HE is the most common cause for possibly preventable re-admissions of patients with liver cirrhosis (Volk et al., 2012).

HE is a serious neuropsychiatric complication of both acute and chronic liver failure with a significant impact on the quality of life and a predictive value of poor outcome (Blei et al., 2008). Although the origin of the toxins responsible for the altered mental has not been yet completely elucidated the role of ammonia remains central supported by a large number of studies (Butterworth, 2002; Ong et al., 2003; Lockwood, 2004; Tarantino et al., 2009).

The occurrence of overt encephalopathy (most of the time hospitalization required) is associated with a survival probability of 42% at 1 year of follow-up and 23% at 3 years (Bustamante et al., 1999). Minimal hepatic (covert) encephalopathy (MHE) is estimated to develop in more than 80% of patients with cirrhosis; Ampuero et al., showed that covert EH is associated with cirrhosis progression, with a higher cumulative and annual incidence rate of disease progression (Ampuero et al., 2018).

HE is the complications of liver cirrhosis (sometimes precipitated by other complications of cirrhosis) that we found in most of the patients hospitalized in acute therapy. Its treatment is often difficult as long as the pathogenic mechanism is not completely clarified.

The diagnosis of EH in a cirrhotic patient is sometimes tricky because neuropsychiatric manifestations can have many causes, the same as in a non-cirrhotic patient. The diagnosis and treatment of MHE, a condition with a high risk of developing overt hepatic encephalopathy (OHE), are essential for the evolution of cirrhotic patients.

2.3.2. Systemic Oxidative Stress Markers in Cirrhotic Patients with Hepatic Encephalopathy: Possible Connections with Systemic Ammoniemia

Background & aim. HE is currently one of the main complications of liver cirrhosis and a characteristic of acute liver failure. Once developed, this neuropsychiatric manifestation of chronic or acute liver disease is associated with high mortality and healthcare costs (Elwir and Rahimi, 2017).

HE is commonly defined as a brain dysfunction secondary to liver insufficiency and/or portosystemic shunting that manifests as a wide spectrum of neurological or psychiatric abnormalities ranging from subclinical disturbances to coma (Aldridge et al., 2015).

Due to its multifactorial, complex and yet unknown pathophysiology, it is thought that HE is generally caused by cerebral edema as a result of the combined action of several factors

such as neurotoxins (e.g., ammonia), impaired neurotransmission following metabolic changes in liver failure, alteration of blood-brain barrier permeability, systemic inflammation, and also oxidative and nitrosative stress (Lemberg et al., 2009; Aldridge et al., 2015).

Although ammonia is widely recognized as the main factor involved in the pathogenesis of HE, the correlation between ammoniemia and HE severity was shown to be significant in acute liver failure by contrast to chronic liver disease. This could suggest that the latter could be influenced also by other pathological factors (Seyan et al., 2010).

The systemic inflammation associated with liver cirrhosis that can trigger the production of reactive oxygen species (ROS) is also able to generate astrocyte swelling and rapid deterioration of neuropsychological function (Cichoz˙-Lach and Michalak, 2013).

There is an increasing number of studies in various animal models that described a possible relevance of oxidative stress as a pathogenic mechanism involved in HE (Kosenko et al., 1997; Robb and Connor, 1998; Kaminski et al., 1999; Kosenko et al., 2003; Norenberg et al., 2004). Although, the implications of oxidative stress in HE pathophysiology are not completely demonstrated, it is clear that there has been an increasing trend in the last 20 years to consider oxidative stress as an important trigger of HE (Seyan et al., 2010; Cichoz˙-Lach and Michalak, 2013).

The aim of our study was to evaluate the role of oxidative stress in the pathogenesis of HE in cirrhotic patients and to establish possible correlations between the systemic oxidative stress markers and the serum ammonia levels.

Material and Methods.

Patients and Healthy Subjects. We conducted a prospective case-control study on cirrhotic patients admitted in a tertiary hospital between April 2018 and June 2018. The study included Child–Pugh class B (15 patients) or C (25 patients) cirrhotic patients, with newly diagnosed overt HE (n = 40, 19 males and 21 females, mean age 56.0 ± 10.4 years).

Exclusion criteria were diagnosis of a neurological or psychiatric disease, anterior diagnosis and/or treatment of HE, current use of psychotropic medications, as well as the absence of signed informed consent. Subjects taking antioxidant supplements were also excluded and also the patients with clinical or biological signs of infection were not included in the study.

The cirrhotic patients were classified according to ammoniemia in two groups. Group A—20 cirrhotic patients with HE and increased systemic ammoniemia and group B—20 cirrhotic patients with HE and normal systemic ammoniemia. The control group consisted of 21 healthy subjects (10 males and 11 females, aged 49.8 ± 9.76 years) matched by age and sex.

The following data have been collected for all patients: demographics, cirrhosis etiology, Child–Pugh class and (MELD) score, medication (used for prevention/treatment of liver cirrhosis and its complications), ammoniemia, oxidative stress markers, HE evaluation (assessed using the West Haven mental status scale).

All subjects or their caregivers gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy of Iasi (no. 19856/03.02.2018).

Measurement of Oxidative Stress Markers. Blood samples were collected following an overnight fasting period during the morning hours at the time of admission and allowed to clot. After centrifugation (3000 rpm, 15 min, 4 ◦C), blood sera were separated, aliquoted and stored at −80 ◦C until subsequent analysis. Serum samples were used to determine serum levels of the oxidative stress markers.

Superoxide dismutase activity (SOD), was measured in a direct dependency manner with reaction inhibition rate (%) of WST-1 substrate (a water-soluble tetrazolium dye) with

xanthine oxidase using a SOD Assay Kit 19160 (Merck, Darmstadt, Germany) from a 40 µl serum sample, according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan) after 20 min of reaction time at 37 ◦C.

Cellular glutathione peroxidase (GPx) activity was measured from a 50 µl serum sample using the GPx cellular activity assay kit CGP-1 (Merck, Darmstadt, Germany). The Glutathione Peroxidase Assay Kit method used an indirect quantification based on coupled enzymatic reactions (GPx-oxidized glutathione-reduced glutathione—glutathione reductase enzymatic chain). During the in vitro assay reactions, the GPx-catalyzed glutathione oxidation is coupled with the glutathione-reductase-catalyzed reverse reaction. Thus, the nicotinamide adenine dinucleotide phosphate consumption can be quantified and due to its direct proportionality, GPx activity can be calculated (PharmaSpec UV-1700 spectrophotometer, Shimadzu, Japan).

Antioxidant enzymes activities were normalized against serum total soluble protein concentration and expressed as enzymatic specific activity units. Therefore, total soluble protein content was measured from 0.2 mL serum sample using Bradford spectrophotometric assay method (PharmaSpec UV-1700, Shimadzu, Japan), in which absorbance at 595 nm was calculated against bovine serum albumin curve, and the results were expressed as mg total soluble proteins/mL serum.

Malondialdehyde (MDA) levels were determined by thiobarbituric acid-reactive substances assay. Serum at a quantity 0.2 mL was added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS–HCl (pH 7.4) and 1 mL of thiobarbituric acid 0.73%. Following vortex mixing, samples were maintained at 100 ◦C for 20 min. Afterward, the samples were centrifuged at 3000 rpm for 10 min and supernatant read at 532 nm (Pharma Spec UV-1700, Shimadzu, Japan). The signal was calculated against an MDA standard curve and the results were expressed as nmols/mL.

Measurement of systemic ammoniemia. Ammoniemia was measured from fresh EDTA-enriched blood samples following centrifugation at 4 ◦C in an integrated automated chemistry system. The ammonia assay reagent is based on glutathione dehydrogenase reaction using a stable analog of nicotinamide adenine dinucleotide phosphate. The analysis was performed according to the manufacturer's instructions and the results were automatically calculated and expressed as µmols/mL. The threshold of high ammonemia was any value above the normal threshold of the hospital laboratory 30–120 mcg/dL. In this regard, the normal ammonemia was considered any value below 120 mcg/dL and high ammonemia all values above 120 mcg/dL.

Statistical Analysis. All statistical tests were performed using the Statistical Package for the Social Sciences software (SPSS version 20; SPSS, Chicago, IL, USA). The Kolmogorov test was used to test the pattern for normal distribution. Continuous variables were expressed as median (interquartile range) and the Mann–Whitney U test or Chi-square test was used to compare parameters. To compare the groups, Student's *t*-test was used for the variables with normal distribution, and the Mann–Whitney U test was used for the variables with asymmetric distribution. Comparisons were carried out by analysis of variance (ANOVA). Correlations between oxidative stress-related markers and serum ammonia levels were analyzed using the Spearman's rank correlation method. Differences were considered statistically significant when the *p*-value was less than 0.05.

Results. *Patient Characteristics.* This study included 40 patients with liver cirrhosis and HE that were classified into subgroups, according to the serum ammonia level (group A—increased systemic ammoniemia and group B—normal systemic ammoniemia) and 21 participants in the control group.

Demographic, clinical and laboratory characteristics of all patients, as well as of those with increased or normal systemic ammoniemia are shown in Table 2.III. Baseline characteristics were generally similar in the two groups. From the liver diseases patients, 24 (60.0%) patients had alcoholic ethology, and 16 (40.0%) had viral etiology (10 with hepatitis C virus-related cirrhosis, and 6 with hepatitis B virus-related cirrhosis). Overall, the median total bilirubin level was 4.03 mg/dL and the median MELD score was 15.0. There were no significant differences between cirrhosis patients with HE and high ammoniemia (group A) and those with normal ammoniemia (group B) regarding gender distribution, cirrhosis etiology, liver disease severity (MELD score, Child–Pugh score), and monitored cirrhosis complications (ascites, spontaneous bacterial peritonitis, upper gastrointestinal bleeding and hepato-renal syndrome), as well as most of the laboratory parameters (Table 2.III). However, group A was characterized by significantly more frequent grade IV hepatic encephalopathy (35.0% vs. 10.0%, $p = 0.043$).

Table 2.III. Demographics, clinical and laboratory parameters patients, according to study groups.

Parameter	All Patients n = 40	Study Group A (High Ammoniemia) n = 20	Study Group B (Normal Ammoniemia) n = 20	p-Value
Gender, Male/Female (%)	19/21 (47.5/52.5)	11/9 (55/45)	8/12 (40/60)	0.342 C
Age, Years, Mean ± SD	56.0 ± 10.4	54.3 ± 9.6	57.6 ± 10.4	0.305 T
Etiology of Cirrhosis, n (%) HCV HBV Alcohol	 10 (25.0) 6 (15.0) 24 (60.0)	 3 (15.0) 3 (15.0) 14 (70.0)	 7 (35) 3 (15.0) 10 (50.0)	0.322 F
Child–Pugh Class B/C, n (%)	15/25 (37.5/62.5)	4/16 (20/80)	11/9 (55/45)	0.022 F
Child–Pugh Score, Median (Q1/Q3)	10 (8/15)	10.5 (9/15)	9 (8/13)	0.077 M
MELD Score, Median (Q1/Q3)	15 (11/20)	21 (11/35)	17 (8/32)	0.356 M
Creatinine (mg/dL), Median (Q1/Q3)	0.78 (0.11/3.5)	0.85 (0.12/2.77)	0.73 (0.11/3.5)	0.810 M
Albumin (g/L), Median (Q1/Q3)	2.8 (1.59/4.5)	2.9 (1.5/3.5)	2.6 (1.96/4.5)	0.394 M
Bilirubin (mg/dL), Median (Q1/Q3)	4.03 (1.74/24.6)	2.15 (1.73/24.1)	4.62 (1.8/16.0)	0.121 M
INR, Median (Q1/Q3)	1.5 (1.28/1.74)	1.42 (1.22/1.63)	1.5 (1.3/1.78)	0.461 M
Ammonia (μmols/L), Median (Q1/Q3)	117 (91.5/188)	158 (113.5/234.5)	69 (60.5/79.5)	0.001 M
Ascites, Mild/Medium/ Large, n (%)	10/14/16 (25/35/40)	5/8/7 (25/40/35)	5/6/9 (25/30/45)	0.536 F
SBP, n (%)	6 (15.0)	2 (10.0)	4 (20.0)	0.096 F
HRS, n (%)	5 (12.5)	3 (15.0)	2 (10.0)	0.633 F
UGIB, n (%)	13 (32.5)	6 (30.0)	7 (35.0)	0.736 F
Encephalopathy, n (%) Stage II Stage III Stage IV	 16 (40.0) 15 (37.5) 9 (22.5)	 9 (45.0) 4 (20.0) 7 (35.0)	 7 (35.0) 11 (55.0) 2 (10.0)	0.043 F

Abbreviations: HBV, chronic hepatitis B virus; HCV, chronic hepatitis C virus; HRS, hepatorenal syndrome; INR, International Normalized Ratio; MELD, Model for End-Stage Liver Disease; SBP, spontaneous bacterial peritonitis; SD, standard deviation; UGIB, upper gastrointestinal bleeding. C Chi-square test; F Fisher exact test; T The t-test (student test); M Mann–Whitney U Test (nonparametric test)

Oxidative Stress-Related Markers. Initial analysis of our results included all liver cirrhosis and newly diagnosed overt HE patients, regardless of their systemic ammoniemia. Biochemical data showed a significant decrease of SOD activity in all subjects with HE, as compared to control group (Table 2.IV) (0.90 ± 0.08 vs 1.35 ± 0.08 U/mL, $p = 0.002$).

Table 2.IV. Oxidative stress markers according to study groups.

Parameter	Controls n = 21	All Cirrhotic Patients n = 40	Study Group A (High Ammoniemia) n = 20	Study Group B (Normal Ammoniemia) n = 20
SOD (U/mL)	1.35 ± 0.08	0.90 ± 0.08	1.06 ± 0.07[a]	0.68 ± 0.08
GPx (U/mL)	0.09 ± 0.006	0.061 ± 0.008	0.08 ± 0.005[a]	0.024 ± 0.004
MDA (nmols/mL)	35.94 ± 1.37	68.90 ± 5.68	76.93 ± 5.48	50.06 ± 5.60

Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; [a] — compensatory increase, as compared to group B

The activity of GPx, was significantly decreased in the HE group compared to healthy age and sex-matched subjects (Table 2.IV) (0.061 ± 0.008 vs. 0.09 ± 0.006 U/mL, $p = 0.01$). In addition, we found that lipid peroxidation marker (MDA) levels were significantly increased ($p = 0.0001$) in the serum of the HE patients, as compared to control group (76.93 ± 5.48 vs. 35.94 ± 1.37 nmols/mL, $p = 0.0001$).

While analyzing the groups separately, by considering systemic ammoniemia (group A and group B), our results showed significant differences in terms of systemic SOD activity (1.06 ± 0.07 in group A vs. 0.68 ± 0.08 U/mL in group B, $p = 0.0002$), suggesting that ammonia could modulate enzymatic activity of SOD.

In this way, statistical analysis revealed significant SOD activity increase in HE patients with normal ammoniemia, as compared to controls (0.90 ± 0.08 vs. 1.35 ± 0.08, $p < 0.0001$), while in HE patients with high ammoniemia SOD activity was decreased, as compared to the control group (1.06 ± 0.07 vs. 1.35 ± 0.08 U/mL, $p = 0.033$). Moreover, our results suggested a significant increase of SOD activity in HE patients and high ammoniemia, as compared to HE patients with normal ammoniemia.

Serum GPx activity was significantly different in all three groups ($F(2.58) = 14$, $p < 0.0001$)) (Table 2.IV). Our results analysis showed a significant GPx activity decrease in HE patients with normal ammoniemia, as compared to controls (0.024 ± 0.004 vs. 0.09 ± 0.006 U/mL, $p < 0.0001$). No significant differences regarding GPx activity were obtained while comparing HE patients with high ammoniemia to healthy age and sex-matched controls (0.08 ± 0.005 vs. 0.09 ± 0.006 U/mL, $p = 0.41$). However, significant increases of GPx activity were noted in HE patients with increased ammoniemia, as compared to HE patients with normal ammoniemia (0.08 ± 0.005 vs. 0.024 ± 0.004 U/mL, $p = 0.0008$).

Regarding the serum MDA levels, the main lipid peroxidation biochemical marker, the initial analysis showed significant group differences ($p < 0.0001$)). Thus, statistical analysis revealed a significant increase of MDA levels in HE patients with normal ammoniemia, as compared to controls (50.06 ± 5.6 vs 35.94 ± 1.37 nmols/mL, $p = 0.007$), as well as a significant increase of MDA concentration in HE patients with high ammoniemia, as compared to control group (76.93 ± 5.48 vs. 35.94 ± 1.37 nmols/mL, $p < 0.0001$).

In addition, a significant increase of serum MDA concentrations were obtained in HE patients with high ammoniemia, as compared with HE patients with normal ammoniemia (76.93 ± 5.48 vs. 50.06 ± 5.60 nmols/mL, $p = 0.017$) (Table 2.IV).

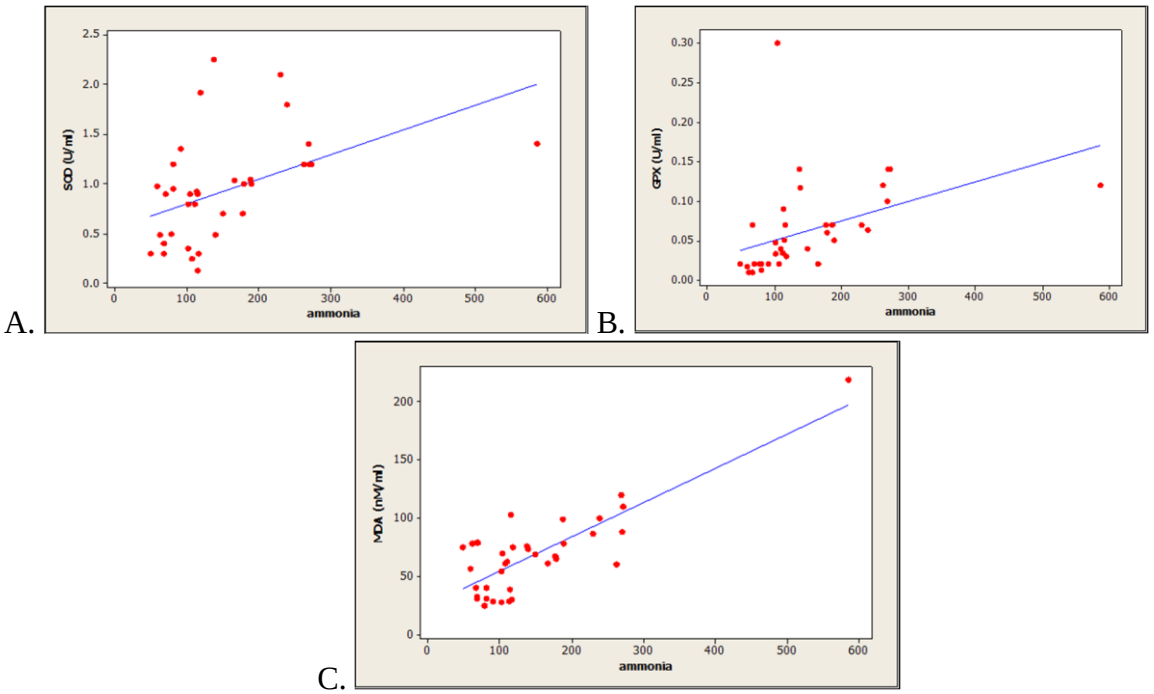


Fig. 2.4. Correlations between oxidative stress markers versus systemic ammonia levels in controls and HE patients (n = 61). (**A**) Superoxide dismutase vs. systemic ammonia levels; (**B**) glutathione peroxidase vs. systemic ammonia levels; (**C**) malondialdehyde vs. systemic ammonia levels.

Correlations between Oxidative Stress-Related Markers and Serum Ammonia Level. Pearson correlation coefficient analysis for all HE patients showed significant direct correlations between the two antioxidant enzymes as follows: SOD vs. ammonia levels (n = 40, r = 0.452, p = 0.005) (Figure 2.4.A), and GPx vs ammonia levels (n = 40, r = 0.460, p = 0.007) (Figure 2.4.B). In addition, we obtained a strong positive correlation between MDA levels and ammoniemia (n = 40, r = 0.855, p = 0.0001) (Figure 2.4.C). However, no significant correlations were found while comparing inflammation marker C reactive protein with ammoniemia (n = 40, r = –0.078, p = 0.733).

Discussion. We found an increased oxidative stress in HE engaged by the significant decrease of serum antioxidant enzymatic activity (SOD and GPx), as well as by the significant increase of MDA concentration, as a lipid peroxidation marker. Moreover, our results are indicating important correlations between systemic ammoniemia and all of the evaluated oxidative stress markers (SOD, GPx and MDA). Also, while evaluating the patients based on their ammoniemia, significant increases of MDA levels were obtained for HE patients with increased ammoniemia, as compared with those with normal ammoniemia. In addition, the increased systemic ammoniemia in HE associated with the present antioxidant enzymes activity trending could suggest the compensatory effect in which, as a result of high oxidative stress (suggested by MDA levels), the antioxidant enzymes try to overcome free radical accumulation and subsequent damage.

However, in normal ammonia levels cirrhotic patients, a high frequency of severe forms (III—55% and IV—10%) was recorded. In this case, not the hyperammonemia theory but the oxidative stress theory could explain the increased occurrence of neurological symptoms. Under normal circumstances as has been portrayed in Figure 2.5, approximatively 90% of the total oxygen is taken up by the mitochondria in order to produce ATP, ROS resulting through the partial four-electron reduction of O_2 to H_2O (Bertram et al., 2006). When the metabolic rate is increased, this is defined by a high production of ROS and is reflected by a depletion of the main antioxidant enzymes, in the present case, SOD and GPx (Birben et al., 2012).

However, the only reliable possibility to decrease ROS generation is to uncouple the metabolic rate (Skulachev VP, 1996).

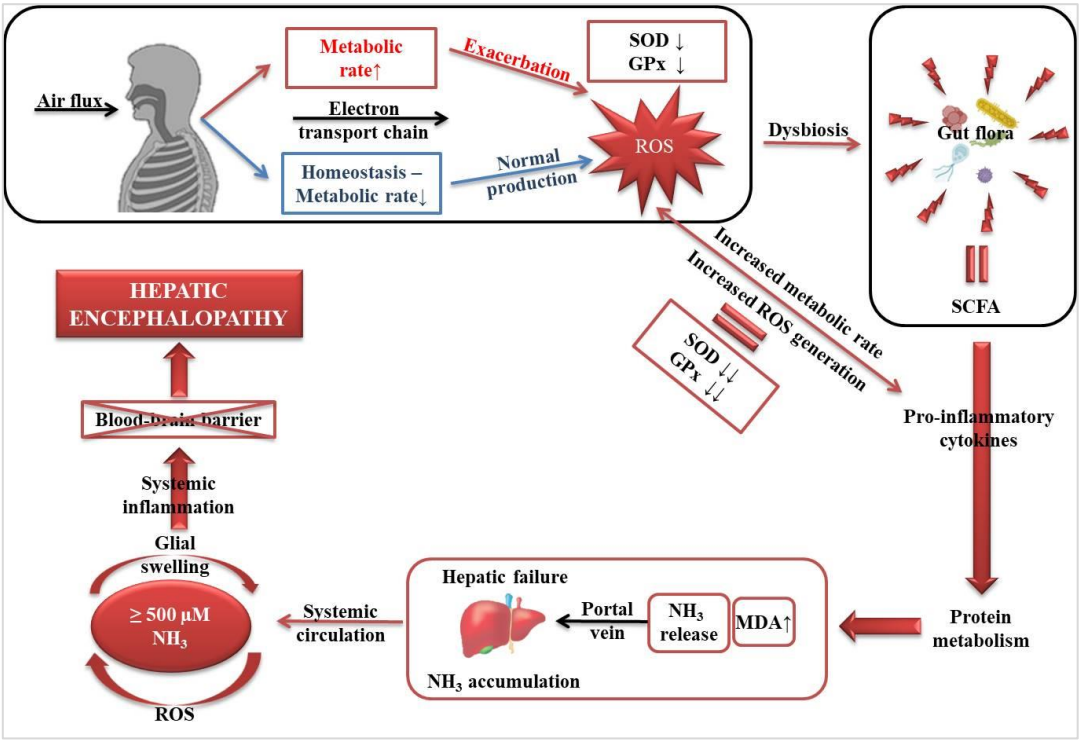


Fig. 2.5. Schematic representation of how oxidative stress gradually disrupts the homeostasis and promotes hepatic encephalopathy.

Regardless of the status of the organism, ROS gradually promotes a dysbiosis (Schwenger, 2019), apart from the imperative mitochondrial dysfunction that exacerbates ROS production (Heidari, 2019). Once the eubiosis is lost, a hazardous environment within the gut microflora emerges and disturbs its entire metabolism (Krishnan et al., 2015; Levy et al., 2017). Subsequently, a series of pro-inflammatory cytokines are activated that further alter protein metabolism (Belizário et al., 2018). Accordingly, the levels of MDA are increased, NH_3 is released and through the portal vein circulates to the liver (Aldridge et al., 2015).

NH_3 enters the systemic circulation and when the levels are higher than 500 µM, systemic inflammation of the glial cells occurs, the blood–brain barrier being unable to act efficiently, which ultimately leads to hepatic encephalopathy (Lockwood, 2004). It must be taken into considerations that this mechanism applies by excluding all harmful exogenous factors.

In our study, we obtained similar results regarding the increased serum MDA concentration, used to assess lipid peroxidation intensity.

An important mechanism that could be described in this context was correlated with hyperamonemia-induced oxidative stress, as some previous studies suggested (Görg et al., 2008; Häussinger and Görg, 2010). Thus, it was shown that the stimulation of N-methyl-D-aspartate receptors by ammonia could result mainly in antioxidant enzymes activity decreasing and superoxide or hydroxylradicals levels increasing (Cichoz˙-Lach and Michalak, 2013). Also, acute astrocytes edema can be implicated in this context, while they could be modulated by the reactive oxygen species and reactive nitrogen species (Reinehr et al., 2007).

Moreover, regarding the relation between ammonia and oxidative stress, the well-known “two-hit hypothesis” of HE could be mentioned. Thus, it states that increased

ammonia, followed by astrocytes dysfunction and oxidative stress is the initial hit. Afterward, the second hit is represented by gastrointestinal bleeding, infection or dehydration-induced ammonia load (Kosenko et al., 1997).

In the present report, we showed that together with the increased oxidative stress status in HE patients' blood serum, strong correlations between systemic oxidative stress markers (two antioxidant enzymes-SOD and GPx and one lipid peroxidation marker-MDA) and ammoniemia were obtained. Moreover, we hereby managed to separately describe the HE patients based on ammoniemia and to report a significant increase of MDA levels in HE patients with increased ammoniemia, as compared to HE patients bearing normal ammoniemia.

Similar studies demonstrated that the increased oxidative stress status is not characteristic to systemic level in HE, as we hereby discuss based on our results, but rather to brain tissues, as in the case of the Gorg et al. study (Görg et al., 2008) that showed that some post-mortem brain cortical tissue oxidative stress markers are increased in HE patients (Irimia et al., 2013). While these oxidative modifications were mainly found in cerebral proteins and cortical RNA, no significant changes of both manganese and copper-dependent SOD activity were recorded.

We showed some variations in SOD activity. Whereas it was significantly decreased in HE patients, as compared to controls, we demonstrated that together with GPx it exhibits a compensatory increase by direct correlation to ammoniemia in HE patients with increased ammoniemia, as compared to HE patients with normal ammoniemia. This was also previously reported by the Singh group (Singh et al., 2008) who described the increased compensatory SOD activity after chronic ammonia-exposure in animal models of HE. Due to the fact that SOD and GPx are critical first-line antioxidant enzymes, it is currently known that they act cooperatively at different sites in the metabolic pathway of free radicals, and could perhaps act as compensatory regulation in response to increased oxidative stress (Halliwell, 2007; Krishna Mohan and Venkataramana, 2007).

While our study strengths were already described and discussed, we should also mention some limitations. Thus, in our best of knowledge, it is only the second prospective study evaluating the influence of serum oxidative stress markers on hospitalized cirrhotic patients with HE in a Romanian tertiary referral center. However, the fact that our study was conducted in single center range by evaluating a small number of patients could be a bias source. Notwithstanding these limitations, we believe our study brings relevant evidence for investigators evaluating the role of oxidative stress in the pathogenesis of hepatic encephalopathy. In addition, we can mention here the lack of a description of spontaneous portosystemic shunts, which could have helped to explain non hyperammonemic encephalopathy.

Conclusions. Our results demonstrated the increased systemic oxidative stress in HE cirrhotics patients, as shown by the significant decrease of both antioxidants enzymes activity, SOD and GPx, as well as by the significant increase of MDA serum levels. Significant correlations between the ammoniemia and all three markers of oxidative stress (SOD, GPx, MDA) were obtained. Also, based on the systemic ammonia levels of the HE patients group division, we observed a significant MDA increase in HE patients with increased ammoniemia, as well as a compensatory increase in the activity of both antioxidant enzymes (SOD and GPx), as compared with HE patients bearing normal ammoniemia. Further research is necessary in order to elucidate the role of oxidative stress in the pathogenesis of HE in cirrhotic patients.

2.3.3. Oral Glutamine Challenge Improves the Performance of Psychometric Tests for the Diagnosis of Minimal Hepatic Encephalopathy in Patients with Liver Cirrhosis

Background & aim. The liver failure and the presence of portosystemic shunts are responsible for increased levels of serum ammonia in patients with liver cirrhosis but the correlations between ammonia levels and the severity of HE have proved to be extremely variable (Blanco Vela et al., 2011). The diagnosis of manifest (overt) EH in a cirrhotic patient with decompensation of liver disease can often be difficult, because all possible causes of neuropsychiatric manifestations, which are quite numerous, must be ruled out.

When cirrhotic patients are found normal under neurological clinical examination, but have mild cognitive and psychomotor deficits, the condition is referred to as MHE. Reaching a diagnosis of MHE can be difficult due to the lack of a gold standard test. Current methods for MHE diagnosis are subjective, time- consuming, costly and hard to access. This might explain why most clinicians do not recommend testing for MHE in current clinical practice.

However, lately, some user-friendly screening tests for evaluating cognitive function in these patients have been proposed. Thus, Psychometric Hepatic Encephalopathy Score, a standardized test battery, has proved to be a short, objective, valid and reliable test for the assessment of MHE (Weissenborn et al., 2001).

Oral glutamine challenge (OGC) is a method to increase blood ammonia in patients with cirrhosis, which could lead to cognitive disturbances. The aim of our work was to evaluate the role of OGC in improving the performance of psychometric tests for the diagnosis of MHE and the risk of this condition for progression to OHE.

Methods. *Patients.* This prospective study included 54 patients diagnosed with liver cirrhosis, hospitalized or followed-up in an outpatient clinic at the Gastroenterology and Hepatology Institute, "St. Spiridon" University Hospital, Iasi, Romania, between March 2010 and June 2011.

Inclusion criteria were: age > 18 years, normal neurologic signs upon examination, stable cirrhosis, grade 0 of HE (West-Haven criteria). Exclusion criteria were: OHE (grade 1 or higher), inability to perform psychometric tests, use of antibiotics, sedatives or lactulose in the previous 3 months, recent (<6 months) active alcoholism, diabetes mellitus, neurologic or psychiatric disorders, evidence of decompensated respiratory, cardiac and renal disease.

All patients underwent upper gastrointestinal endoscopy to detect the presence of esophageal varices. Arterial ammonia blood level assessment, together with the psychometric tests were performed pre- and post-glutamine load. Each patient was followed-up every 4 months over one year for development of OHE.

The study was conducted according to the provisions of the Helsinki Declaration and was approved by the local Ethics Committee. All subjects gave their written informed consent.

The control group consisted of 16 healthy subjects matched to patients according to age, sex, and education level.

Psychometric tests. After a thorough explanation of the tests, each patient and healthy subject performed the PHES at baseline and at 60 minutes after OGC. Psychometric Hepatic Encephalopathy Score includes number connection tests A and B, digit-symbol test, line tracing test and serial dotting test. The results of psychometric hepatic encephalopathy score were determined using an online free calculator, available at http://www.redeh.org/TEST_phes.htm, based on the normality tables in the Spanish population. The diagnosis of MHE was defined by a Psychometric Hepatic Encephalopathy Score value $\leq$ -5.

Oral glutamine challenge (OGC). In a fasting state, patients and healthy control subjects ingested a 20g solution of glutamine (L-glutamine, Prolab Nutrition Inc. USA) dissolved in 100 ml tap water. Any symptoms that appeared during one hour were recorded.

Samples of arterial blood (radial puncture) were taken at baseline and 60 minutes after glutamine load. Blood samples were immediately placed on ice and transported within 30 minutes to the laboratory for centrifugation and ammonia determination.

Data analysis. Statistical analysis was performed using Medcalc 12.3.0 and Microsoft Office Package. The results were expressed as means ± SD. We used the receiver operating characteristics (ROC) curve in order to analyze the sensibility and the specificity of blood ammonia level (pre- and post-glutamine) as a tool for the diagnosis of MHE and for establishing optimal threshold values. Correlations between variables were examined with a Pearson correlation. Multivariate regression was performed for the determination of individual predictive factors for OHE.

Results. The demographic and clinical characteristics of the patients are summarized in Table 2.V.

Table 2.V. Characteristics of the studied patients

Patients (n = 54)	
Mean age, years (range)	55.2 ± 7.6 (42 – 63)
Gender (M/F)	34 / 20
Etiology of cirrhosis	
Alcohol	34
HCV	16
HBV	2
Cryptogenic	2
MELD score (range)	9 (7 – 19)
Child-Pugh class (A/B/C)	31/19/4
Esophageal varices (grade I/II/III)	8/10/12

Ammonia blood levels. Post-glutamine load, a significant raise of arterial ammonia levels in patients with cirrhosis (85.2±20.8 versus 159.82±66.01 µg/dL, p <0.0001) was observed, while in healthy control subjects the changes did not reach the level of significance (47.15±17.3µg/dL *vs.* 52.15±18.07µg/dL, p =0.064).

Using the ROC curve analysis, cut-off values of ammonia blood levels both at baseline and post-OGC were defined (87.8 µg/dL and 124µg/dL, respectively). For the diagnosis of MHE, baseline blood ammonia showed an area under the ROC curve (AUROC) of 0.54 (CI: 0.402-0.680, p= 0.58), while the post- OGC was 0.53 (CI: 0.389-0.667, p=0.77).

Prevalence of MHE before and after OGC. At baseline, 29 of 54 patients (53.7 %) met the psychometric hepatic encephalopathy score criteria for MHE diagnosis. After glutamine load, the percentage of patients diagnosed with MHE increased to 43 (79.6 %). The values of psychometric hepatic encephalopathy score were significantly lower post-OGC compared to baseline (P<0.0001), suggesting that OGC increased the diagnostic performance of psychometric hepatic encephalopathy score for MHE in cirrhotic patients, and it remained almost unchanged in healthy subjects. An altered OGC defined by ROC curve as an increase of ammonia over 124 µg/dL post-glutamine load was found in 37 patients (68.51%). Among these, 30 (81.1%) had MHE, while 7 (18.9%) did not have MHE.

Incidence of overt HE during the follow-up. On follow-up, 10 patients (18.51%) developed OHE. Among these, 9 had MHE (4 at baseline and 5 after glutamine load), and 1 patient met no criteria for MHE both at baseline and post-glutamine.

The development of OHE was significantly associated with the post-glutamine psychometric hepatic encephalopathy score (n= 54, r= -0.382, p= 0.004), while PHES alone did not show any significant correlation (n= 54, r= -0.140, p= 0.313).

The following variables were considered in a predictive model for OHE: Child and MELD scores, grade of esophageal varices, pre- and post-glutamine arterial blood ammonia.

In the multivariate regression analysis only the MELD score was an independent predictive factor for the development of OHE (OR = 1.5187, 95% CI: 1.0690 – 2.1574, p = 0.0197).

Discussion. HE is the late effect of portal hypertension with a high grade of porto-systemic shunts, spontaneous or surgically created. MHE has no specific clinical expression, thus being hard to assess. Diagnosis of MHE is still controversial, especially when it comes to establish criteria and reliable diagnostic tests easily applied in clinical practice. Neurological and psychological evaluations do not offer sufficient clinical tools for diagnosing MHE and assessing its progression to OHE.

In the present study, we found that glutamine load increases the performance of psychometric tests for MHE diagnosis in patients with liver cirrhosis. Previous studies have reported that glutamine-induced increased levels of ammonia were associated with variable modifications of psychometric testing (Romero-Gomez et al., 2002; Oppong et al., 1997; Ditisheim et al., 2011; Masini et al., 2003). In another study (Masini et al., 2003) the authors showed that increased level of ammonia following glutamine load did not change psychometric testing results for attention, orientation and memory. Douglass et al (Douglass et al., 2001), using a combination of aminoacids with a hemoglobin-like composition, created a model of episodic encephalopathy and showed significant changes in ammonia concentration as well as in psychometric and electroencephalographic testing. Increased concentrations of ammonia were also reported in patients with cirrhosis Child A, without inducing any modifications in the psychometric testing performance. The differences in psychometric testing results throughout reports in the literature could be explained by variations in the type of test used, diagnosis criteria, and the type and dose of glutamine. Using the battery of psychometric hepatic encephalopathy score, which is accepted as a standard diagnostic test for MHE in several countries (Schomerus et al., 1999; Romero Gomez et al., 2006; Amodio et al., 2008; Marks et al., 2008), we showed that it represents an efficient tool in the evaluation of cirrhotic patients with mild cognitive deficits.

Glutamine was administrated at a dose of 20 g, similar to that in some studies (Romero Gomez et al., 2006), but higher than what was being used in others. However, this dose was well tolerated and did not precipitate OHE in our cirrhotic patients. In line with previous reports (Romero-Gomez et al., 2002; Masini et al., 2003; Ditisheim et al., 2011), glutamine load did not modify the psychometric testing results and ammonia levels in the controls, demonstrating that it is not implicated in the cognitive status of the healthy subjects.

In our study, the prevalence of MHE at baseline was 53.7%, which is concordant with previous studies (Das et al., 2001; Sharma and Kumar, 2002; Gomez et al., 2006), but higher than in other studies that included patients with mild or moderate hepatic disease (Quero et al., 1996; Groeneweg et al., 2000). Ditisheim et al. (Ditisheim et al., 2011) reported a prevalence of 44% for MHE in patients with moderate or severe hepatic failure. These differences suggest the implication of multiple risk factors in MHE, besides the severity of hepatic failure (Groeneweg et al., 2000). Thus, alcoholic etiology of cirrhosis in 62.96% of cases and large esophageal varices (3rd degree) in 22.22 % of patients could be major risk factors for MHE in our study.

We decided to perform OGC using arterial blood ammonia as, according to previous studies (Snady and Lieber, 1988; Kramer et al., 2000), it proved more relevant than the venous one in the assessment of HE. Our study showed that arterial ammonia does not represent a specific biological marker for the diagnosis of MHE. Moreover, glutamine does not improve its diagnostic performance, as showed by the AUROC value (0.54 at baseline and 0.53 post- glutamine load).

It seems that assessing ammonia level in capillary blood could be a simpler and more convenient alternative to arterial determination (McCullough, 1969; Huizenga et al, 1995).

In the present study, the incidence of OHE on the follow- up was 18.51%, a result in line with some previous reports (Romero-Gomez et al., 2002). The risk for OHE development was significantly higher in patients with MHE than in those without MHE. This fact confirms previous data regarding the natural history of MHE and its role in the progression to OHE.

Post-glutamine psychometric hepatic encephalopathy score, unlike psychometric hepatic encephalopathy score alone, was significantly associated with the development of OHE (p= 0.004), showing that OGC increases its prognostic value. We have also found that the MELD score was a predictive factor with an independent value for OHE development. Considering the increase in both diagnostic performance and prognostic value of post-glutamine psychometric hepatic encephalopathy score, this test could be a useful and better screening tool for MHE than PHES alone. However, its feasibility is limited by the lack of convenience and time constraints.

Our study has several limitations such as the relatively small size of the samples, especially of patients with severe liver disease. Since psychometric hepatic encephalopathy score evaluation has not yet been standardized in our country, we have used a standardized psychometric hepatic encephalopathy score scoring based on Spanish norms which could influence our classification. However, the presumed differences between norms of other countries were limited by the use of a control group. Future studies should definitely move towards finding a larger applicable set of norms for psychometric hepatic encephalopathy score scoring in representative samples.

Conclusions. In cirrhotic patients, an oral glutamine load improves the performance of psychometric tests for the diagnostic of MHE, a condition which has proved to have a high risk for development of OHE. In addition to MHE, MELD score is an independent predictive factor for the development of OHE.

Volk, et al. showed that EH is the most common cause for possibly preventable readmissions of patients with liver cirrhosis (Volk et al., 2012). If we analyze the factors that could reduce the recurrence of HE and of readmissions we notice the very often lack of communication/education and continuous supervision of the patients.

In an older study we found that upper gastrointestinal bleeding, infectious complications and alcohol intake were the most important precipitants factors of HE in patients with liver cirrhosis. Some factors are difficult to be avoided but the dietary protein, diuretic therapy and alcohol intake could be corrected resulting a low incidence of HE (Cojocariu et al, 2005).

Updating this data would be important to re-evaluate the spectrum of EH precipitating factors.

2.4. INFECTIOUS COMPLICATIONS IN PATIENTS WITH LIVER CIRRHOSIS

2.4.1. Introduction

The incidence of bacterial infections in cirrhotic patients is still very high and affects the number and duration of hospitalizations and sometimes even life expectancy. 25–35% of patients were diagnosed with bacterial infections when entering the hospital or developed this complication during hospitalization. The incidence is 4–5 fold higher than that observed in the general population (Fernández et al., 2012; Angeli et al., 2018). More importantly, the incidence of bacterial infections in cirrhotic patients is one of LC complications in which the mortality is still high and has not changed substantially and are directly responsible for 30–50% of deaths in cirrhosis.

The most common infections in cirrhotics are spontaneous bacterial peritonitis (SBP) and urinary tract infection, followed by pneumonia skin and soft tissue infections, and bacteremia (Angeli et al., 2018). Poor liver function, VB, low ascitic fluid protein levels, prior

SBP and hospitalization are the most clinical factors associated with an increased risk of infection (Gustot et al., 2009; Fernandez and Gustot, 2012). Infectious complications increases 3.75 fold death risk in patients with decompensated cirrhosis, reaching 30% at 1 month and 63% at 1-year (Foreman et al., 2003; Arvaniti et al., 2010).

In 2011 we found that the incidence of bacterial infections in cirrhotic patients is high (30% in admitted patients) and 24,5% of them occurred during hospitalization. The most frequently complication were urinary (no significant mortality in the absence of cumulative risk factors) and SBP with high mortality despite intensive treatment (Cojocariu et al., 2011).

2.4.2. The Risk of *Clostridioides difficile* Infection in Cirrhotic Patients Receiving Norfloxacin for Secondary Prophylaxis of Spontaneous Bacterial Peritonitis

Background & aim. SBP is a major complication of LC associated with high mortality. It is occurred in 10–30% of adult cirrhotic patients with ascites and has an in-hospital mortality rate of 20–40% (Ginés et al., 1990; Fiore et al., 2019).

Norfloxacin is a quinolone with limited absorption from the gastrointestinal tract which has antibacterial activity against Gram-negative bacteria. It has been reported that norfloxacin determines gut decontamination and has high efficacy in SBP secondary prophylaxis in patients with LC (Ginés et al., 1990; Fernández et al., 2006).

The EASL (Angeli et al., 2018) and AASLD (Runyon, 2013) guidelines recommend that cirrhotic patients with previous episodes of SBP should receive long-term secondary prophylaxis with norfloxacin (400 mg/day) as long as they have ascites. The efficacy of this treatment was first demonstrated by Gines et al who reported that the use of norfloxacin as secondary prophylaxis for SBP decreased the recurrence of SBP from 68% to 20% (Ginés et al., 1990). However, there are still controversies regarding the safety of long-term use or norfloxacin. Thus, the European Medicines Agency has released a warning on quinolone antibiotics recommending a restriction in the use of these drugs because the possible side effects as tendiniti, tendon rupture, hyperglycemia, and aortic aneurysm or dissection (European Medicines Agency. Antibiotics, 2019). In addition, bacterial resistance should be a major concern in cirrhotic patients, that most of them had previous hospitalizations and a high probability of being colonized by multidrug resistant bacteria. Moreover, in the recent years, an alarm sign has been raised on the long-term safety of this treatment regarding the risk of *Clostridium difficile* infection (CDI). The associated CDI may have a major impact on the outcome of cirrhosis including higher mortality rates (Bajaj et al., 2010**)**.

These data suggest that the use of quinolones for SBP secondary prophylaxis in patients with LC should be reconsidered. The aim of this study was to evaluate the incidence and the risk factors for CDI development in patients receiving long-term norfloxacin for secondary prophylaxis of SBP.

Materials and Methods. *Patients.* In this prospective observational cohort study we included all consecutive patients with liver cirrhosis who were hospitalized for SBP between January 2018 and December 2019 for SBP in a tertiary university hospital. All the patients were successfully treated for SBP. On the day of hospital discharge, all patients received the recommendation for secondary prophylaxis of SBP.

We excluded patients with hepatocellular carcinoma, those who died during hospitalization, patients receiving immunosuppressive drugs, and those with human immunodeficiency virus infection, known hypersensitivity or intolerance to norfloxacin, previous seizure, prior transjugular intrahepatic portosystemic shunting, prior solid organ transplantation, prior episodes of SBP, or associated illnesses with a life expectancy of 1 month or who could not be regularly followed-up. All the patients were evaluated every 6 months or whenever it was imposed by the LC complications.

Liver cirrhosis diagnosis was based on clinical, laboratory and imaging findings. The severity of liver cirrhosis was graded according the Child-Pugh class and MELD score. Presence of ascites was based on clinical findings and confirmed with abdominal ultrasound. Comorbidities were recorded for all the patients. All the patients diagnosed with SBP were treated according to the recent guidelines (Runyon, 2013; Angeli et al., 2018). At discharge the patients included in the study received norfloxacin 400 mg/day as secondary prophylaxis of SBP as much as they have ascites.

Clostridioides difficile infection. CDI was diagnosed based on the presence of more than three watery stools within 24 hours plus the presence of *Clostridium difficile* (*C. difficile*) toxins A and/or B (enzyme immunoassay) in stool samples.

Community-associated CDI was defined as the onset of CDI outside a healthcare facility or within 48 hours following admission to a healthcare facility without contact from a healthcare facility within the previous 12 weeks (Cohen et al., 2010; Rao and Malani, 2020).

Nosocomial CDI was defined as CDI with onset of symptoms: on day three or later, following admission to hospital or in the community within four weeks of discharge from a healthcare facility (Rao and Malani, 2020).

Recurrent CDI was defined as diarrheal stools with a positive laboratory test after the end of treatment more than 2 weeks and less than 8 weeks following the onset of a previous episode (Mullish et al., 2018; Cohen et al., 2010).

In all patients, an informed written consent to use their clinical data for scientific purposes was systematically obtained at the entry. The study was conducted in accordance with the Declaration of Helsinki and was approved by the internal review board and Ethical Committee (number 1284/24 November 2017).

Statistical analysis. Categorical variables were expressed as frequency and percentage. Continuous variables were expressed as mean plus/minus standard variation for normally distributed continuous data, and as median and range (25th to 75th percentile) to describe non-normally distributed continuous data. Groups were compared using $\chi 2$ test for categorical variables and using independent *t* test or Mann-Whitney *U* test for continuous variables (depending on data distribution). Univariate analysis was performed for each recorded data. Variables with $P< 0.1$ in univariate analysis were included in multivariate analysis (Cox regression). Odds ratio (OR) with 95% confidence interval (CI) was calculated for qualitative variables included in the logistic regression. P value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

Results. A total of 2520 patients with liver cirrhosis were admitted to our tertiary hospital during the study period, of whom 272 (10.8%) were diagnosed with SBP and only 122 with first episode of SBP were eligible for the study. Most of the patients were males (65.5%), mean age 57.5±10.8 years and had alcoholic liver cirrhosis (63.1%). Baseline characteristics of the patients included in the study are presented in Table 2.VI. The majority of the patients had large ascites (62.3%) and hepatic encephalopathy (85.2%). The mean MELD score was 19.7±6.1 and Child-Pugh class C patients (69.7%) were the most prevalent.

All the patients included in the study were diagnosed with SBP, the majority (105 patients -86.1%) had community-acquired SBP. All the patients received secondary SBP prophylaxis with norfloxacin 400 mg/day as long as they had ascites. Most of the patients received non-selective beta-blockers (72.1%) and only 26 patients (21.3%) had chronic treatment with proton pomp inhibitors (PPIs). The median follow-up during the treatment period was 7 months.

Of all the patients included in the study 23 patients (18.8%) developed CDI during follow-up.

Table 2.VI. Baseline characteristics of the study groups.

Parameter	All Patients n = 122	Patients with CDI n = 23	Patients without CDI n = 99	p-Value
Age, years, Mean ± SD	57.5 ± 10.8	57.8 ± 11.8	57.4 ± 10.7	0.874
Mean follow-up, months, Median, (IQR)	7 (2–15)	7 (2.5–15.5)	7 (2–14.5)	0.638
Gender, male, n (%)	80 (65.5)	20 (86.9)	60 (60.6)	0.017
Etiology of cirrhosis, n (%) Alcohol HBV HCV	 77 (63.1) 26 (21.3) 19 (15.6)	 18 (78.3) 5 (21.7) 0 (0)	 59 (59.6) 21 (21.2) 19 (19.2)	0.030
Child-Pugh class, n (%) B C	 37 (30.3) 85 (69.7)	 3 (13.1) 20 (86.9)	 34 (34.3) 65 (65.7)	0.045
Child-Pugh score, Median, (IQR)	10 (9–12)	12 (10–12.5)	10 (9–12)	0.016
MELD, Mean ± SD	19.7 ± 6.1	21.6 ± 7.9	19.3 ± 5.5	0.095
Grade 3 ascites, *n* (%)	76 (62.3)	18 (78.2)	58 (58.6)	0.079
ALT, UI/L, Median, (IQR)	33 (24–65)	44 (31–66)	32 (24–63)	0.290
Platelets × 105, Median, (IQR)	118 (78–142)	100 (76–128)	122 (79–144)	0.046
INR, Median, (IQR)	1.62 (1.4–1.95)	1.9 (1.47–2.36)	1.6 (1.38–1.9)	0.059
Total bilirubine, mg/dl, Median, (IQR)	4.2 (1.79–7.72)	7.28 (2.18–8.44)	4.06 (1.79–7.08)	0.203
Albumin g/dl, Median, (IQR)	2.19 (1.93–2.77)	2.26 (1.98–2.9)	2.17 (1.9–2.7)	0.282
Creatinine mg/dl, Median, (IQR)	0.79 (0.64–1.22)	0.81 (0.76–1.53)	0.77 (0.63–1.18)	0.211
CRP, g/dl, Median, (IQR)	2.9 (1.8–6.7)	2.7 (2.1–7.9)	2.9 (1.7–6.1)	0.736
SBP recurrence, *n* (%)	18 (14.8)	6 (26.1)	12 (12.1)	0.089
Death, *n* (%)	66 (54.1)	14 (60.8)	52 (52.5)	0.496
PPIs, *n* (%)	26 (21.3)	5 (21.7)	21 (21.2)	0.956
BBs, *n* (%)	88 (72.1)	18 (78.2)	70 (70.7)	0.467
Rifaximin, *n* (%)	104 (85.2)	20 (86.9)	84 (84.8)	0.797
Number of admissions during follow-up, Mean ± SD	2.27 ± 1.48	1.91 ± 1.41	2.36 ± 1.50	0.192
Antibiotic treatment during follow-up, n(%)	12 (9.8)	2 (8.7)	10 (10.1)	0.838
Total proteins ascites, Median, (IQR)	1.1 (0.8–1.6)	1.1 (0.8–1.46)	1.1 (0.8–1.65)	0.760

CDI: *Clostridioides difficile* infection; SD: standard deviation; IQR: interquartile range; HBV: hepatitis B virus; HCV: hepattis C virus; MELD: Model for End-Stage Liver Disease; ALT: alanine aminotransferase; INR: International Normalized Ratio; CRP: C-reactive protein, SBP: spontaneous bacterial peritonitis; PPIs: proton pump inhibitors; BB: betablockers

The overall CDI incidence rate was 24.8 cases per 10,000 person-years. Three patients were diagnosed with healthcare-associated CDI. They were diagnosed with CDI during hospitalization for hepatic encephalopathy. Six patients (26.1%) had recurrent form of CDI. All the patients received treatment with vancomycin 125 mg every 6 hours orally for 10 days. In ten patients the dose of vancomycin was increased to 250 mg every 6 hours as they did not response to the initial dose. Four patients with recurrent CDI received the tapering vancomycin regimen.

There was no significant difference in the presence of hepatic encephalopathy or hepato-renal syndrome, the concomitant use of beta-blockers or PPIs as well as for most of the laboratory parameters between cirrhotics that developed CDI and those without CDI (Table 2.VI). The majority of the patients (85.2%) received rifaximin for hepatic encephalopathy prophylaxis. However, the patients that developed CDI during follow-up were predominantly males (86.9% vs. 60.6%, P=0.017), had significantly higher Child-Pugh score (12 points vs. 10 points, P=0.016) and more frequent alcoholic etiology of LC (78.3%

vs. 59.6%; P=0.030). No previous reported quinolones side effects were identified in our cohort.

Eighteen patients (14.8%) had SBP recurrence during follow-up. The development of CDI infection did not influence SBP recurrence rate. During follow-up 66 patients (54.1%) died. The mortality rate was similar in patient with or without CDI (60.8% vs. 52.5%, P=0.496) (Figure 2.6).

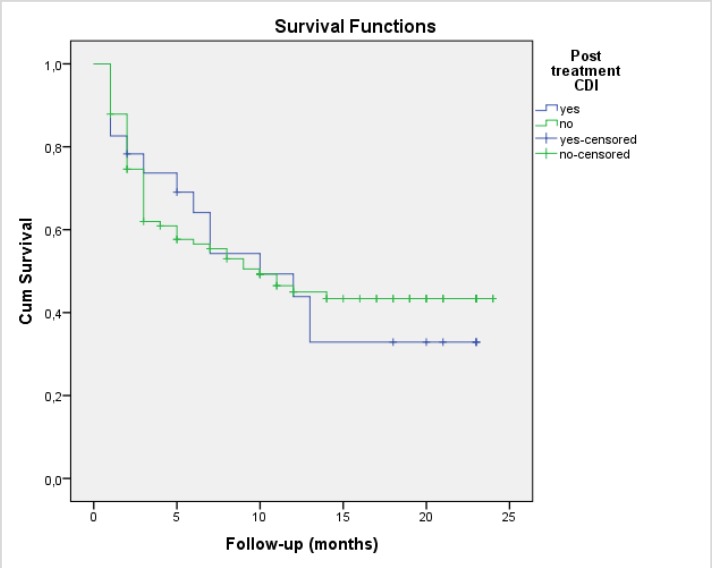


Figure 2.6. Survival rate in patients with and without CDI

The results of the univariate and multivariate logistic regression analyses are shown in Table 2. The males with alcoholic LC, Child-Pugh C class and large ascites were at risk to develop CDI during follow-up. The multivariate Cox regression analysis demonstrated that alcoholic LC etiology (HR 3.18, 95% CI 1.104-2.441, P=0.029) and Child-Pugh C class (HR 2.50, 95% CI 1.257-3.850, P=0.034) were independent risk factors for CDI development during norfloxacin secondary prophylaxis for SBP (Table 2.VII).

Table 2.VII. Risk factors for CDI -univariate and multivariate analyses.

Parameter	Univariate Analysis			Multivariate Analysis		
	OR	95%CI	p-Value	HR	95%CI	p-Value
Alcoholic etiology	1.38	1.082–1.775	0.030	3.18	1.104–2.441	0.029
Child-Pugh C class	1.32	1.070–1.639	0.033	2.50	1.257–3.850	0.034
Age > 65 years	1.23	0.558–2.715	0.615			
Males	1.43	1.147–1.795	0.011			
Grade 3 ascites	1.33	1.018–1.753	0.045			
Comorbidities	0.56	0.273–1.153	0.069			
Ascitic liquid proteins < 1.5 g/L	1.01	0.724–1.406	0.959			
Rifaximin	1.02	0.857–1.226	0.795			
PPIs	1.10	0.862–1.421	0.458			

CDI: *Clostridioides difficile* infection; OR: odds ratio; CI: confidence interval; HR: hazard ratio; PPIs: proton pump inhibitors

Discussion. Previous studies have evaluated the incidence of CDI in patients with liver cirrhosis and concluded that it is higher than in the general population (Bajaj et al., 2010). Patients with LC are prone to develop infection complications especially in the advanced for of this disease. Previous hospitalization, the immunocompromised system, the comorbidities

and most important the previous antibiotic treatment were the main risk factors for CDI development in patients with LC. It was also demonstrated that the development of CDI in cirrhotic patients is associated with an increased risk of mortality, prolonged hospitalization and higher hospitalization costs (Bajaj et al., 2010).

SBP is a severe complication of LC associated with a high risk of mortality. The secondary prophylaxis with norfloxacin has proven to be effective in these patients (Ginés et al., 1990). The gut decontamination by eliminating the aerobic gram-negative bacilli reduce the rate of SBP recurrence caused by *Enterobacteriaceae* (Marciano et al., 2019). Norfloxacin is the quinolone that has been mostly used because of 72on o72e solubility, low permeability, and therefore low bioavailability, characteristics that theoretically allow it to selectively decontaminate the bowel. Moreover in vitro studies demonstrated that norfloxacin could have an anti-inflammatory effect by decreasing the level of TNF alfa, explaining the positive effect on mortality (Dalhoff, 2005; Gómez-Hurtado et al., 2011; Juanola et al., 2016).

Even if the efficacy of this treatment was clearly demonstrated, questions regarding the safety of this treatment were raised recently. Several Food and Drug Administration (FDA) Drug Safety Communications (U.S. Food and Drug. FDA in Brief: FDA Warns That Fluoroquinolone Antibiotics Can Cause Aortic Aneurysm in Certain Patients. 2018. Available online: https://www.fda.gov/news-events/fda-brief/fda-brief-fda-warns-fluoroquinolone-antibiotics-cancause-aortic-aneurysm-certain-patients (accessed on 2 April 2021) preceded the European Medicines Agency alert (European Medicines Agency. Antibiotics. 2019). They highlighted the association between fluoroquinolone/quinolone use and significant hypoglycemia and, side effects involving the tendons, muscles, joints, and nerves with an increased risk of developing tendinitis and tendon rupture. Moreover, recently published works evidenced an increased incidence of aortic aneurysm or dissection secondary to quinolones use (Lee et al., 2015; Pasternak et al., 2018). Considering all these side effects concerns have been raised regarding the safety of long-term administration of norfloxacin in patients with LC. As for, the aim of our study was to evaluate the incidence and the risk factors associated with the CDI development in patients with LC receiving norfloxacin as secondary prophylaxis of SBP.

In our cohort the incidence of CDI cirrhotic patients was 24.8 cases per 10,000 person-year, lower that the incidence reported in a cohort of cirrhotic patients with HE (Stoica et al., 2015). Previous studies demonstrated that in general population treated with antibiotics 5% of the patients developed CDI (Borzio et al., 2001), in our cohort 6.7% developed CDI during norfloxacin secondary SBP prophylaxis. It should be also mentioned that the majority of the studies on CDI epidemiology in LC were retrospective 72on o72e different methods of CDI diagnosis, with a high risk of underestimating the real incidence of this disease (Trifan et al., 2015). Most of the patients developed CDI infection after 6 months of norfloxacin prophylaxis and the presence of CDI did not influence the mortality. The patients with alcoholic liver cirrhosis and Child-Pugh class C had a significant higher risk to develop CDI during follow-up. Sundaram et al. Also reported a significantly higher prevalence of CDI among patients with alcoholic liver disease compared to those without alcoholic liver disease (1.62 % vs. 1.04 %, $P<0.001$) (Sundaram et al., 2014) and these data were confirmed by our study. The recurrence rate was only 14.8% comparable with the data previously reported (Yan et al., 2019). It has to be mentioned that most of the patients included in our study received also rifaximin for hepatic encephalopathy prophylaxis. Even if Bajaj et al demonstrated that cirrhotic patients with CDI have a higher mortality rate and length of hospital stay compared with those without CDI, in our cohort the CDI infection did not influence the mortality rate. These data are comparable with some data obtained in patients with LC without SBP (Sundaram et al., 2014).

Most of the randomized controlled trials did not report CDI as complication of long-term antibiotic prophylaxis for variceal hemorrhage (Fernandez, 2006) or SBP (Kemp et al., 2009, Lontos et al., 2014; Marciano et al., 2019), more over norfloxacin was associated with reduced of *C. difficile* in patients stools (Gines et al., 2009). Although, when compared to other antibiotic class, not to placebo, norfloxacin was associated with CDI in 9.7% of cases compared 73on o cases in the co-trimoxazol group (Jafferbhoy et al., 2011).

Our real-world data partially confirmed the data from randomized controlled trials. In the randomized controlled trials Norfloxacin was found to reduce the probability of recurrence of SBP as Gines et al demonstrated (Gines et al., 2009; Moreau et al., 2018) these data were confirmed by our study, although the risk of CDI still remained in our cohort.

Our study has some strengths and also has several limitations. Thus, it is the first study that had the primary outcomes the evaluation of the incidence and the risk factors for developing CDI in cirrhotic patients receiving long-term norfloxacin prophylaxis for SBP. However, as a single center study it is more likely to produce bias secondary to the small number of cases or underdiagnosed CDI in some cases. In addition, the study provides no information on the *C. difficile* strain.

Conclusions. The patients with Child-Pugh class C alcoholic liver cirrhosis have a high risk of developing CDI during long-term norfloxacin treatment for SBP secondary prophylaxis. For these patients alternative prophylaxis should be evaluated. SBP secondary prophylaxis should be personalized in order to outweigh the risks associated with the CDI development.

Only a few studies have been conducted concerning factors influencing infectious complications so in a prospective study we aimed to assess the incidence of infection in decompensated hospitalized cirrhotic patients and to evaluate possible risk factors for this complication. We showed that patients who developed an infection were more likely to have a high MELD score, to be hospitalized for hepatic encephalopathy, to stay in the intensive care unit, and to undergo invasive procedure. Our study indicates that patients with severe cirrhosis who are hospitalized for hepatic encephalopathy and receive corticosteroid treatment have a higher risk of developing a bacterial infection during their hospitalization than other cirrhotic patients (Girleanu et al., 2017).

2.5. ACUTE-ON-CHRONIC-LIVER-FAILURE – ANOTHER CHALLENGE IN PATIENTS WITH LIVER CIRRHOSIS

2.5.1 Introduction

LC the end stage of any chronic liver disease generally is characterized by a long asymptomatic evolution; the diagnosis is frequently established in the setting of decompensation and/or complications such as VB, ascites, HE, acute kidney injury (AKI), and bacterial infections which associate a poor prognosis (Hernaez et al., 2017; Das et al., 2010). Moreover, the practitioners have been often surprised by rapid impairment of liver, kidney or circulatory function, in patients with known, yet stable liver cirrhosis. These observations suggested the existence of a distinct syndrome, different from the classic decompensation of liver cirrhosis, namely acute-on-chronic liver failure (ACLF). ACLF is a relatively recently recognized syndrome that is different from LC and acute liver failure in terms of outcome.

Jalan R et al. were the first described ACLF in 2002 (Jalan and Williams, 2002): but since then many definitions have been proposed, most of them lacking solid clinical proof, which led to a discrepancy between the prevalence and clinical outcomes of ACLF reported by different authors (Katoonizadeh et al., 2010; Bajaj, 2013).

Several international societies and consortia have attempted to characterize this syndrome however, a universally accepted definition has not yet been found. The latest definition was enunciated by The Asian Pacific Association for the Study of the Liver (APASL), defining ACLF as jaundice and coagulopathy complicated within four weeks by clinical ascites and/or encephalopathy in a patient with a previously diagnosed or undiagnosed chronic liver disease/cirrhosis, associated with a high 28-day mortality, following an acute hepatic injury (Sarin et al, 2019). In one of our studies we found at 28 days an appreciable mortality in patients with ACLF, ranging from 24% in ACLF 1 stage to 91.7% in 3 stages; 90-day mortality was between 56% in ACLF 1 stage and 98% in 3 stage (Chiriac, 2017). The conditions associated with ACLF vary from hepatitis B reactivation, super infection with hepatitis E, bacterial fungal or parasitic infections, drug-induced liver injury, alcohol abuse, autoimmune hepatitis flare, acute variceal bleeding, and also relative adrenal insufficiency (Trifan et al., 2013; Sarin et al., 2019).

Moreau et al. (Moreau et al., 2013) conducted the first large prospective multicentric study, carried on in Europe (the CANONIC study) in which the authors defined ACLF as acute decompensation of liver cirrhosis associated with organ failure and high short-term mortality rates. The authors established specific criteria for the diagnosis of the syndrome. The authors diagnosed ACLF in 30.9% of the participants included. I will return to this data later; but I must point out that we diagnosed ACLF in 68.8% of patients admitted to our department, a value significantly higher than in other studies and which certainly needs to be analyzed and discussed (Chiriac, 2017).

In order to define organ failure and to assess disease severity several scores have been proposed, namely the Chronic Liver Failure Consortium Organ Failure (CLIF-C OF) score, a simplified version of the CLIF-C ACLF score (Arroyo et al., 2015).

Certain precipitating factors of ACLF have been identified, such as active alcohol consumption, and bacterial infection which were the most common; gastrointestinal bleeding, therapeutic paracentesis without administration of intravenous albumin, placement of trans jugular intrahepatic portosystemic shunt, major surgery, viral hepatitis, and alcoholic hepatitis could precipitate ACLF. Surprisingly, in 40.3% of the cases no precipitating event was identified, and the outcome was not influenced by the precipitating factor but rather by the number of organ failures. The most commonly identified failing organ in ACLF is the kidney; also, studies indicate that ACLF is associated with younger age and alcohol abuse and patients without any history of decompensation developed a more severe syndrome (Moreau et al., 2013).

My experience in this pathology is proven primarily by the clinical activity which often involves the management of patients with ACLF. Moreover, we have published the first data about the real-life perception of ACLF in Romania – survey filled in by practicians (Chiriac et al. 2019). This questionnaire was applied in order to raise awareness of the existence/importance of ACLF. In other study we aimed to assess the prognostic value of ammonia in patients with ACLF in terms of in-hospital mortality.

2.5.2. Real-life perception of ACLF in Romania: results of a survey completed by practicians

Background & aim. The practitioners have been often surprised by rapid impairment of liver, kidney or circulatory function, in patients with known, yet stable liver cirrhosis.

These observations suggested the existence of a distinct syndrome, different from the classic decompensation of liver cirrhosis, named ACLF, often unrecognized. The aim of our study was to assess the patterns of practice, disease awareness and recognition of ACLF among Romanian practitioners.

Materials and methods. We composed a survey based on the results of the CANONIC study (Moreau et al., 2013) and consisting of questions regarding the current knowledge on ACLF (Table 2.VIII). Most questions were dichotomous, and the questionnaire was designed to be as short and as little time consuming as possible.

Table 2.VIII. The survey used for the evaluation of the practitioners

General information regarding the participants	
1. Please choose your specialty	□ gastroenterology □ intensive care □ internal medicine
2. Please choose your professional degree	□ intern □ specialist □ consultant
3. Are you currently working in a university hospital?	□ yes □ no
Knowledge of ACLF	
1. Have you herd of ACLF?	□ yes □ no
2. In your opinion, do you consider ACLF to be different from the acute decompensation of liver cirrhosis?	□ yes* □ no
Diagnostic criteria for ACL	
1. Do you consider that currently there are unanimously accepted criteria for the diagnostic of ACLF?	□ yes □ no*
2. When you suspect the presence of ACLF, do you find the CLIF-OF score useful in establishing the diagnosis?	□ yes* □ no
3. Is liver failure mandatory for the diagnosis of ACLF?	□ yes □ no*
4. Is the degree of ascites relevant for the diagnosis of ACLF?	□ yes □ no*
Particularities of ACLF	
1. Is liver failure the most commonly found organ dysfunction in ACLF patients?	□yes □ no*
2. Do younger or alcoholic patients pose a higher risk of developing ACLF than the others?	□ yes* □ no
3. Is ACLF evolution more severe in patients with previous acute decompensation?	□ yes □ no*
4. Is mortality in patients with ACLF similar of higher with those associating acute decompensation of liver cirrhosis?	□ yes* □ no

*correct answer, ACLF=acute-on-chronic liver failure

The survey was sent by e-mail to the contact list of members of the Romanian Society of Gastroenterology and Hepatology (SRGH); additional printed survey were sent to intensive care practitioners from "Sf. Spiridon" County Clinical Emergency Hospital Iași, Romania. The questionnaire was divided into several sections; the first section included questions regarding to participant information (age, sex, place of practice, the professional degree, the affiliation to a university hospital). We emphasized gathering information regarding the participant's clinical and diagnostic abilities concerning the assessment of ACLF; thus, we distributed the questions according to the following criteria: knowledge of ACLF, diagnostic criteria of ACLF, and particularities in patients with ACLF. The survey was sent to the participants in March 2017. The responses were received from April to September 2017. The results of survey were expressed as percentage.

We were also interested in comparing our results with unpublished data presented by Trifan, et al. on 4th Up Date on Hepatology Course, April 6th-7th 2017, Bucharest, Romania (Figure 2.7). We were also interested in observing if the presentations held on ACLF during the meeting had an effect on the general concepts of Romania practitioners.

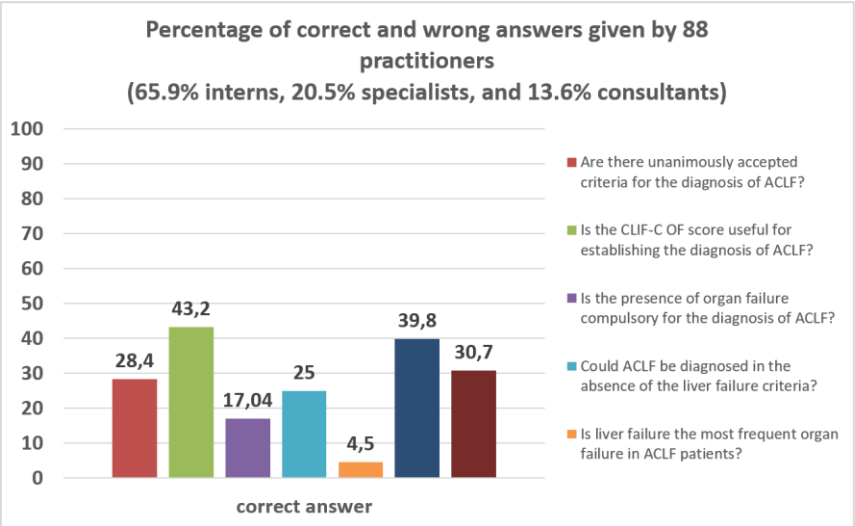


Fig. 2.7. Real-life perception of ACLF in Romania: results of a survey completed by practicians in a northeastern Romanian university tertiary care center

Results. Out of 317 gastroenterology practitioners, members of SRGH, 181 (57%) responded. All of the 24 intensive care specialists that received the printed version of the survey responded. Out of 72 internal medicine consultants 58 (80%) responded. The total number of respondents was 263, and included 181 (69%) gastroenterologists, 58 (22%) internal medicine specialists and 24 (9%) intensive care specialists. Regarding the professional degree, 82 (31%) of responders were trainees, 92 (35%) were consultants and 89 (34%) were senior consultants.

Most practitioners worked in a university hospital (66%) and the majority of the respondents said that they had heard of ACLF (94%) and seventy eight percent of them considered ACLF to be different from the "simple" acute decompensation of liver cirrhosis. 191 (73%) of responders considered that there were unanimously accepted criteria for the diagnosis of ACLF.

183 (70%) of responders considered the CLIF-OF score is useful in establishing the diagnosis of ACLF. When asked what organ dysfunction was the most common among patients with ACLF, almost all of them (98%) found liver failure to be the most commonly organ insufficiency. How-ever, 41% of them stated that severe liver failure was not mandatory for the diagnosis of this syndrome. The degree of ascites was not considered relevant for the diagnosis of ACLF by more than half of the practitioners (65%) and a great proportion agreed that the younger and alcoholic patients had a higher risk for developing ACLF (84%). Data obtained from the survey is illustrated in Table 2.IX.

Table 2.IX. Results of the survey conducted

Question	Yes (%)	No (%)
1. Have you heard of ACLF?	94	6
2. Do you consider ACLF to be different from the acute decompensation of liver cirrhosis?	78	22
3. Do you consider that currently there are unanimously accepted criteria for the diagnosis of ACLF?	73	27
4. When you suspect the presence of ACLF, do you find the CLIF-OF score useful in establishing the diagnosis?	70	30
5. Is liver failure mandatory for the diagnosis of ACLF?	59	41
6. Is the degree of ascites relevant for the diagnosis of ACLF?	35	65
7. Is liver failure the most commonly found organ dysfunction in ACLF patients?	98	2

Question	Yes (%)	No (%)
8. Do younger or alcoholic patients pose a higher risk of developing ACLF that the others?	84	16
9. Is ACLF evolution more severe in patients with previous acute decompensation?	94	16
10. Is mortality in patients with ACLF similar or higher with those associating acute decompensation of liver cirrhosis?	76	24

Discussion. ACLF was firstly mentioned by Sarin (Sarin et al., 2018) and for a long time an evidence-based definition of ACLF was not established. The syndrome was characterized based on expert opinions and not on objective data. In 2014 more than13 definitions of ACLF were accepted (Wlodzimirow et al., 2013).

According to WGO Consensus Definition ACLF is a syndrome distinct from acute liver failure or decompensated cirrhosis in patients with chronic liver disease (with or without known cirrhosis), characterized by acute hepatic decompensation (liver failure and one or more organ failures), associated with high mortality within 28 days and up to 3 months from onset (Jalan et al., 2014).

ACLF is a newly defined concept, but our survey confirms that the majority of the Romanian practitioners have heard about this entity (94%) without significance depending on the specialty. Unfortunately, although having heard of ACLF most of practitioners do not know whether there are unanimously accepted criteria for the diagnosis of ACLF. Trifan et al., on The 4th UpDate on Hepatology Course, 6th-7th April 2017, Bucharest, Romania reported that 27.6% of practitioners in a North-eastern Romanian university tertiary center were aware of unanimously accepted criteria for the diagnosis of ACLF. Our survey revealed that 73% of responders were confident with the diagnosis criteria. Diagnosis criteria for ACLF represent a subject still under debate and the answers of our responders show relative uncertainty, in accordance with the lack of unanimously accepted diagnostic criteria.

ACLF is defined as acute decompensation of liver cirrhosis associated with organ failure and high short-term mortality rates. The diagnosis is made in the setting of organ failure(s), as defined by the CLIF-C OF, a score developed by Moreau et al. by adapting the Sequential organ failure assessment (SOFA) score (used by all intensive care practitioners) in accordance with the particularities of cirrhotic patients. One hundred and eighty-three of the responders (70%) considered the CLIF-C ACLF score useful in establishing the diagnosis of ACLF. The percent of correct responses is significantly higher compared to the percent reported by Trifan et al. in April 2017 during the 4th Update on Hepatology Course Bucharest, Romania. Unfortunately, we have no data of current evaluation of CLIF-C OF score (except for intensive care units) in daily clinical practice.

ACLF should be assessed in all patients with acute decompensation of cirrhosis: ascites grade 2 to 3 (according to definition of International Ascites Club) within less than 2 weeks (first/new episode), acute hepatic encephalopathy in patients with previous normal consciousness (first/new episode), acute upper and/or lower gastrointestinal hemorrhage of any etiology and bacterial infections (any type of acute bacterial infection) in patients with liver cirrhosis. ACLF should not be assessed in patients with refractory ascites or chronic hepatic encephalopathy.

Although many of us do not use this score we emphasize that it is not very time consuming (easy access http://www.efclif. com/ scientific-activity/score-calculators/clif-c-aclf.) and not more time consuming than calculating the Child or MELD scores, requiring no special equipment and there-fore could find its place in clinical practice.

Conclusions. The prevalence of ACLF in Romania is currently unknown, but the mortality rate of patients with liver cirrhosis is known to be high, as while liver cirrhosis is the fourth cause for death in a recent WHO report with a mortality rate of 47.8/100,000 (World

Health Organization. Global status report on alcohol and health 2014). ACLF is a newly syndrome that benefits from increased attention; thus, we conducted a survey in a representative population of Romanian practitioners in order to assess the current knowledge regarding this entity and to set the grounds for future prospective studies in our country. The results show that knowledge of the diagnosis and characteristics of ACLF among practitioners in Romania are according with the current ACLF published criteria. However, future research is needed in order to evaluate the percent of practitioners that apply the ACLF criteria in clinical practice.

After we reviewed the results of the study, we realized that it would be useful to see if our warning about ACLF changed medical practice and even more so if it improved the prognosis of these patients.

A national study on ACLF would certainly bring important data about the profile of our patients, the particularities of approach and treatment. As a first objective we aim to analyze the incidence of ACLF in our Institute and then to propose a study model that includes all patients with ACLF in Romania.

2.5.3. Role of ammonia in predicting the outcome of patients with ACLF

Background & aim. Hyperammonemia has I as a consequence of the reduction in the detoxification capabilities of the liver in the setting of liver failure as well as with increased intestinal production of ammonia (Jaeger et al., 2019).

Although ammonia is widely recognized as the main factor involved in the pathogenesis of HE, the association with the severity of HE is much clearer in acute liver failure than in chronic liver disease, suggesting that in the latter there might be other factors involved, such as systemic inflammation that could lower the threshold for neurological impairment caused by ammonia (Seyan et al., 2010). Patients with acute liver failure and high ammonia levels may experience cerebral edema, followed by subsequent herniation and death (Liotta and Kimberly, 2020). However, the relation between high ammonia levels and HE in patients with ACLF has not been adequately described. Although there is data indicating hyperammonemia as a potential mechanism for HE from experimental ACLF models, there are only a few studies to demonstrate this relation in clinical settings (Seyan et al., 2010). Moreover, there is still conflicting data concerning the risk of in hospital death for patients with ACLF and high ammonia levels. While several studies found that hyperammonemia was associated with increased mortality (Kumar et al., 2012; Shalimar et al., 2019).

This study aimed to assess the prognostic value of ammonia in patients with ACLF in terms of in-hospital mortality.

Materials and methods. *Study design.* We conducted a retrospective study on patients hospitalized between January 2017 and January 2019. We included consecutive cirrhotic patients admitted for non-elective indications such as ascites, HE, upper gastrointestinal bleeding, or bacterial infections that fulfilled the APASL diagnostic criteria of ACLF. Patients diagnosed with non-hepatic malignancy, human immunodeficiency virus infection, hematological disease as well as pregnant women were excluded from the study. LC was diagnosed based on clinical criteria, laboratory tests, typical imagistic findings, and upper digestive endoscopy consistent with portal hypertension.

The end-point of the study was considered in-hospital death. Patient electronic records were analyzed and information regarding sex, demographics, etiology of liver disease, duration of hospitalization and the in-hospital death rates was recorded. The abdominal ultrasound and upper digestive endoscopy results were obtained. Laboratory tests from fasting venous blood taken at admission were noted. Complete blood count, serum transaminase levels, total serum bilirubin, albumin, creatinine, international normalized ratio (INR), lactate, C-reactive protein (CRP), sodium, and ammonia levels were retrieved.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy of Iasi. There was no requirement for the informed written consent because of the retrospective nature of the study; all of the patients signed an informed consent upon hospitalization agreeing to receive treatment.

Definitions. ACLF was defined according to APASL criteria (the online calculator available at http://www.aclf.in/) and ACLF grade was established accordingly. APASL definition for ACLF was chosen because electronic records from our database did not contain information regarding the PaO_2 or the FiO_2 and thus the European Foundation for the Study of Chronic Liver Failure Consortium definition criteria for ACLF could not be evaluated. HE was defined using the West Haven criteria (Dharel and Bajaj, 2015). Acute kidney injury was defined according to the International Club of Ascites definition (Khwaja, 2012).

Statistical analysis. IBM SPSS version 22.0 was used for the statistical analysis. Continuous variables were expressed either as mean ± SD or as median (interquartile ratio), according to the parametric or non-parametric distribution. The Kolmogorov–Smirnov test was used to assess the distribution. The Student's t-test, Chi-square or Fischer's exact tests were used for the analysis of continuous and categorical variables respectively. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used for the assessment of accuracy in predicting the outcome for Child-Pugh, model for end-stage liver disease (MELD), AARC scores and venous ammonia (VA). The cohort was then divided and further analyzed according to the cut-off value obtained for VA. Univariate and multivariate analysis was performed to assess the risk factors for in hospital mortality. The statistical methods of the study were reviewed by a biomedical statistician.

Results. Five hundred and twenty patients were screened. After applying the exclusion criteria 74 patients were removed from further analysis, thus 446 patients were included in the study. The median age of the participants was 59 (50-65) years and 57.4% of the patients were men. The main etiology of LC was alcohol (78.7%), followed by HCV infection (11.2%), HBV infection (6.1%), alcohol and HBV (1.8%), alcohol and HVC (1.3%), HBV and HVC (0.7%). The patients' general characteristics are presented in Table 2.X.

Most of the patients had advanced liver disease, as indicated by the high values of the traditional Child-Pugh and MELD prognosis scores of 11 (10-12) and 19.13 ± 6.79 respectively. The median novel AARC score was 7 (6-8), consistent with the predominance of grade I ACLF found in the majority of the patients; 66.4% of the participants had ACLF grade I, 31.2% ACLF grade II, and 2.5% ACLF grade III. Noncontrast computed tomography cranial scans were performed in 34 patients and no evidence of cerebral edema was described.

Table 2.X. General cohort characteristics

Variable	Value
Age, median (IQR)	59 (50-65)
Male sex, *n* (%)	256 (57.4)
Etiology of liver disease, *n* (%) Alcohol Hepatitis C virus Hepatitis B virus Alcohol + hepatitis B virus Alcohol + hepatitis C virus Hepatitis B and C virus	 351 (78.7) 50 (11.2) 28 (6.1) 8 (1.8) 6 (1.3) 3 (0.7%)
Albumin (g/dL), mean ± SD	2.38 ± 0.59
Bilirubin (mg/dL), median (IQR)	2.53 (1.41-4.82)
INR, median (IQR)	1.47 (1.29-1.74)
Platelets ($\times 10^9$/L), median (IQR)	128 (90-178)

Variable	Value
WBC ($\times 10^9$/L), median (IQR)	7.41 (5.45-10.36)
CRP (mg/dL), median (IQR)	1.51 (0.82-3.1)
Venous ammonia (μmol/L), median (IQR)	103 (78-148)
Sodium (mmol/L), median (IQR)	134 (130-137)
Creatinine (mg/dL), median (IQR)	0.815 (0.63-1.27)
Lactate (mmol/L), median (IQR)	1.7 (1.2-2.1)
Child-Pugh score, median (IQR)	11 (10-12)
Child-Pugh class, *n* (%) A B C	 3 (0.7) 84 (19.2) 350 (80.1)
MELD, mean ± SD	19.13 ± 6.79
AARC score, median (IQR)	7 (6-8)
ACLF, *n* (%) Grade I Grade II Grade III	 296 (66.4) 139 (31.2) 11(2.5)
Ascites, *n* (%) Grade I Grade II Grade III	408 (91.5) 37(9.1) 112 (27.5) 259 (63.5)
HE, *n* (%) Grade I Grade II Grade III Grade IV	374 (83.9) 127 (34) 139 (37.2) 88(23.5) 20(5.3)
IQR: Interquartile range; SD: Standard deviation; INR: International normalized ratio; WBC: White blood cells; CRP: C-reactive protein; MELD: Model for end-stage liver disease; AARC score: APASL ACLF Research Consortium score; HE: Hepatic encephalopathy; GI: Gastrointestinal; UTI: Urinary tract infection; CDI: Clostridium Difficile infection; AKI: Acute kidney injury; SBP: Spontaneous bacterial peritonitis; HCC: Hepatocellular carcinoma	

Mortality analysis. In-hospital mortality was 7.8% (Table 2.XI). The mean survival of the deceased patients was 5 (3-10) days. The causes of death were: multi-organ failure in 19 patients, severe HE in 9 patients, and hemorrhagic shock in 7 patients. Non-survivors had higher levels of bilirubin, higher INR, lower albumin, and consequently higher Child-Pugh and MELD scores, consistent with more advanced liver disease than survivors. However, a higher inflammatory response expressed through higher CRP and higher white blood cells (WBC) count was also noted in the deceased, independently of the presence of bacterial infections, thus suggesting the important role of an exacerbated inflammatory response in the prognostic of patients with ACLF and HE. As expected, most of the non-survivors had grade II and III ACLF (62.9%, 22.9% respectively), while most of the survivors had grade I ACLF (70.8%).

Table 2.XI. Mortality analysis

Variable	Survivors, *n* (%) 411 (92.2)	Non-survivors, n (%) 35 (7.8)	p-Value
Age, median (IQR)	59 (50-65)	60 (49-66)	0.956
Male sex, *n* (%)	239 (58.2)	17 (48.6)	0.271
Albumin (g/dL), mean ± SD	2.4 ± 0.59	2.16 ± 0.5	0.022
Bilirubin (mg/dL), median (IQR)	2.33 (1.37-4.67)	3.52 (1.93-6.49)	0.013

Variable	Survivors, *n* (%) 411 (92.2)	Non-survivors, n (%) 35 (7.8)	p-Value
INR, median (IQR)	1.45 (1.27-1.68)	1.81 (1.5-2.6)	0.005
Creatinine (mg/dL), median (IQR)	0.82 (0.63-1.26)	0.77 (0.62-1.32)	0.397
Platelets ($\times 10^9$/L), median (IQR)	132 (92-180)	123 (82-173)	0.481
WBC ($\times 10^9$/L), median (IQR)	7.24 (5.37-9.89)	11.04 (6.79-13.45)	0.014
CRP (mg/dL), median (IQR)	1.36 (0.8-2.98)	3.15 (1.7-4.67)	0.001
Venous ammonia (μmol/L), median (IQR)	100 (75-139)	198 (125-345)	< 0.001
Venous ammonia > 152.5 μmol/L, *n* (%)	75 (19)	24 (70.6)	< 0.001
Sodium (mmol/L), median (IQR)	134 (130-137)	133 (130-137)	0.215
Child-Pugh score, median (IQR)	11 (10-12)	13 (11-15)	< 0.001
Child-Pugh score ≥ 12.5	58 (14.4)	19 (57.6)	< 0.001
Child-Pugh class, *n* (%)			
A	3 (0.7)	0	
B	84 (20.8)	0	
C	317 (78.5)	33 (100)	
MELD score, mean ± SD	18.43 ± 6.26	27.31 ± 7.37	< 0.001
MELD score ≥ 22.5	119 (29)	27 (77.1)	< 0.001
AARC score, median (IQR)	7 (6-8)	9 (9-11)	< 0.001
AARC score ≥ 8.5	42 (10.2)	26 (74.3)	< 0.001
ACLF, *n* (%)			
Grade I	291 (70.8)	5(14.3)	
Grade II	117 (28.5)	22 (62.9)	
Grade III	3 (0.7)	8(22.9)	
Ascites, *n* (%)	376 (91.5)	32 (91.4)	0.991
HE, *n* (%)	341 (83)	33 (94.3)	0.081
Grade I	126 (37)	1 (3)	
Grade II	135 (39.6)	4 (12.1)	
Grade III	76 (22.3)	12 (36.4)	
Grade IV	4 (1.2)	16 (48.5)	

IQR: Interquartile range; SD: Standard deviation; INR: International normalized ratio; WBC: White blood cells; CRP: C-reactive protein; MELD: Model for end-stage liver disease; AARC score: APASL ACLF Research Consortium score; HE: Hepatic encephalopathy; GI: Gastrointestinal; AKI: Acute kidney injury; SBP: Spontaneous bacterial peritonitis; HCC: Hepatocellular carcinoma

HE was diagnosed in 94.3% of the non-survivors and in 83% of the survivors (P =0.081). However, 84% of the deceased had severe HE (grade III or IV), compared to 23.5 % of the survivors (P < 0.001). Overall VA was higher in the deceased than in the survivors' group [100 (75-139) μmol/L vs 198 (125-345) μmol/L, P < 0.001]. ROC analysis showed a slightly better accuracy for the prediction of in-hospital mortality for the AARC score (AUC = 0.886) than for the MELD score (AUC = 0.816) and the VA (AUC = 0.812). The accuracy of the Child-Pugh score in predicting mortality was fair (AUC = 0.799) (Figure 2.8).

Subsequently, cut-off values for the prediction of mortality were identified for VA (152.5 μmol/L, sensitivity = 0.706, 1-specificity = 0.190), AARC score (8.5, sensitivity =0.743, 1-specificity = 0.102), MELD score (22.5, sensitivity = 0.771, 1-specificity = 0.286), and Child-Pugh score (12.5, sensitivity = 0.576, 1-specificity = 0.144). Univariate analysis found that AKI [odds ratio (OR) = 5.636, confidence interval (CI) (2.634-12.063)], severe HE (grade III or IV) [OR = 18.270, CI (6.830-48.870)], VA ≥ 152.5 μmol/L [OR = 7.976, CI (3.950-16.104)], MELD score ≥ 22.5 [OR = 8.282, CI (3.657-18.752)], Child-Pugh score ≥ 12.5 [OR = 8.096, CI (3.846-17.041)], and AARC score ≥ 8.5 [OR = 25.381, CI (1.151-57.770)] was associated with in-hospital mortality. However, multivariate analysis identified AARC score ≥ 8.5 and VA ≥ 152 μmol/L to be independent predictors of in-hospital mortality (Table 2.XII).

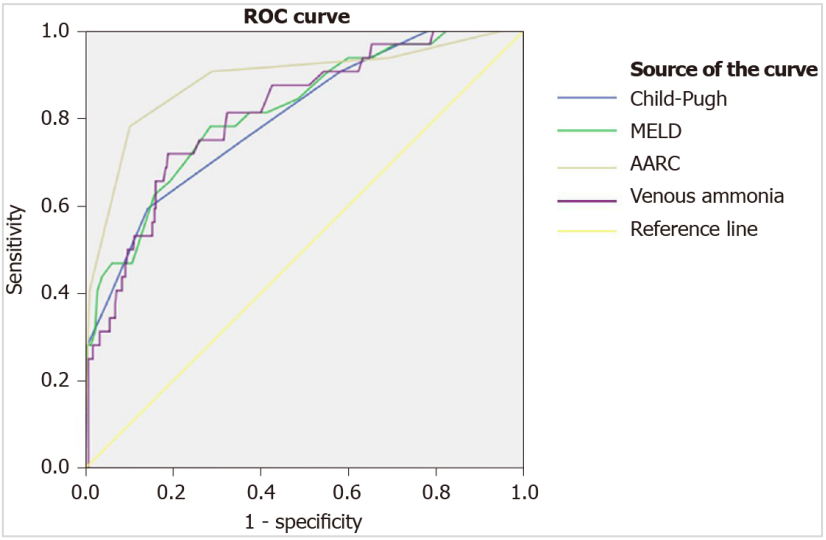


Fig. 2.8. Receiver operating characteristics curve analysis. Diagonal segments are produced by ties. ROC: Receiver operating characteristics; MELD: Model for end-stage liver disease; AARC: Asian Pacific Association for the Study of the Liver Acute-on-chronic Liver Failure Research Consortium.

Table 2.XII. Multivariate analysis of the predictive factors for in-hospital mortality

Variables in the equation								
							95%CI for EXP (B)	
Step 11	**B**	**SE**	**Wald**	**df**	**Sig**	**Exp (B)**	**Lower**	**Upper**
AKI	0.593	0.577	1.055	1	0.304	1.809	0.584	5.607
MELD score ≥ 22.5	0.815	0.610	1.787	1	0.181	2.259	0.684	7.463
Child-Pugh score ≥ 12.5	0.366	0.568	0.415	1	0.520	1.442	0.473	4.394
AARC score ≥ 8.5	1.849	0.642	8.305	1	0.004	6.354	1.807	22.349
HE grade III or IV	1.154	0.628	3.383	1	0.066	3.172	0.927	10.853
Venous ammonia ≥ 152 μmol/L	-1.813	0.508	12.763	1	0.000	0.163	0.060	0.441
Constant	-2.795	1.368	4.172	1	0.041	0.061		

HE and high VA analysis. HE was diagnosed in 83.9% of patients, with 34% having grade I, 37.2% grade II, 23.5% grade III, and 5.3% grade IV HE. Patients with HE had lower levels of albumin and higher levels of bilirubin, MELD, Child-Pugh and AARC scores. Moreover, Patients with HE presented more often ascites and AKI, as a consequence of the higher severity of their liver disease (Table 2.XIII).

Table 2.XIII. Comparison of patient characteristics based on the presence of hepatic encephalopathy

Variable	**HE, *n* (%) 374 (83.9)**	**No. HE, *n* (%) 72 (16.1)**	**p-Value**
Age, median (IQR)	60 (50-65)	56 (47-65)	0.413
Male sex, *n* (%)	211 (56.4)	45 (62.5)	0.339
Albumin (g/dL), mean ± SD	2.33 ± 0.55	2.60 ± 0.74	< 0.001
Bilirubin (mg/dL), median (IQR)	2.62 (1.54-5.11)	1.72 (0.73-3.30)	0.015
INR, median (IQR)	1.49 (1.31-1.75)	1.34 (1.14-1.57)	0.054
Creatinine (mg/dL), median (IQR)	0.82 (0.63-1.26)	0.77 (0.62-1.32)	0.520
Platelets (× 10^9/L), median (IQR)	127 (90-173)	154 (93-193.5)	0.054
WBC (×10^9/L), median (IQR)	7.5 (5.41-10.45)	7.12 (5.76-9.09)	0.898
CRP (mg/dL), median (IQR)	1.53 (0.83-3.15)	1.29 (0.72-2.24)	0.795
Venous ammonia (μmol/L), median (IQR)	111 (86-158)	74 (60-93)	< 0.001
Venous ammonia > 152.5 μmol/L, *n* (%)	96 (26.6)	3 (4.5)	< 0.001
Sodium (mmol/L), median (IQR)	134 (129-137)	135 (131-138)	0.883
Child-Pugh score, median (IQR)	11 (10-12)	9 (8-11)	< 0.001
Child-Pugh class, *n* (%)			

Variable	HE, *n* (%) 374 (83.9)	No. HE, *n* (%) 72 (16.1)	p-Value
A	1 (0.3)	2 (3)	
B	47 (12.7)	37 (55.2)	
C	322 (87)	28 (41.8)	
MELD, mean ± SD	19.74 ± 6.88	15.97 ± 5.24	< 0.001
AARC score, median (IQR)	7 (6-8)	9 (9-11)	< 0.001
ACLF, *n* (%)			
Grade I	224 (59.9)	72(100)	
Grade II	139 (37.2)	0	
Grade III	11 (2.9)	0	
Ascites, *n* (%)	337 (90.1)	71 (98.6)	0.018
Upper GI bleeding, *n* (%)	71 (19)	18 (25)	0.247
SBP, *n* (%)	23 (6.1)	1 (1.4)	0.150
Other infections, *n* (%)	64 (17.1)	6 (8.3)	0.061
AKI, *n* (%)	51 (13.6)	1 (1.4)	0.003
HCC, *n* (%)	10 (2.7)	1 (1.4)	0.520
Hospital stay (d), median (IQR)	8 (6-11)	5 (3-10)	0.061
In-hospital death rate, *n* (%)	33 (8.8)	2 (2.8)	0.081

IQR: Interquartile range; SD: Standard deviation; INR: International normalized ratio; WBC: White blood cells; CRP: C-reactive protein; MELD: Model for end-stage liver disease; AARC score: APASL ACLF Research Consortium score; HE: Hepatic encephalopathy; GI: Gastrointestinal; AKI: Acute kidney injury; SBP: Spontaneous bacterial peritonitis; HCC: Hepatocellular carcinoma

VA median value was 103 (78-148) μmol/L, with higher levels in the HE than in the non-HE groups [111 (86-158) vs 74 (60-93), respectively, P < 0.001]. When further analyzing the subgroup of patients with high VA levels (≥ 152 μmol/L), significant differences were found concerning the distribution of cases with HE, severe HE, and ascites. Patients with high VA levels had a more advanced liver disease, as shown by the high MELD and AARC scores and a significantly higher in-hospital death rate (Table 2.XIV).

Table 2.XIV. Comparison of patient characteristics based on the presence of high venous ammonia

Variable	Venous ammonia > 152.5 μmol/L, n (%) 99 (23.1)	Venous ammonia ≤ 152.5 μmol/L, n (%) 329 (76.9)	p-Value
Age, median (IQR)	57 (48-65)	60 (50-65)	0.481
Male sex, *n* (%)	58 (58.6)	190 (57.8)	0.883
Albumin (g/dL), mean ± SD	2.33 ± 0.54	2.38 ± 0.59	0.452
Bilirubin (mg/dL), median (IQR)	2.7 (1.48-5)	2.33 (1.37-4.7)	0.230
INR, median (IQR)	1.5 (1.31-1.83)	1.46 (1.27-1.72)	0.584
Creatinine (mg/dL), median (IQR)	0.85 (0.63-1.54)	0.81 (0.63-1.18)	0.397
Platelets ($\times 10^9$/L), median (IQR)	121 (73-173)	134 (93-182)	0.134
WBC ($\times 10^9$/L), median (IQR)	8.39 (5.251-11.02)	7.26 (5.47-9.92)	0.359
CRP (mg/dL), median (IQR)	1.4 (0.84-4.16)	1.5 (0.8-2.98)	0.682
HE, *n* (%)	265 (80.5)	96 (97)	< 0.001
HE grade III or IV, *n* (%)	55 (20.8)	49 (51)	< 0.001
Sodium (mmol/L), median (IQR)	134 (131-137)	134 (129-137)	0.970
Child-Pugh score, median (IQR)	11 (10-12)	11 (10-12)	0.173
Child-Pugh class, *n* (%)			
A	1 (1)	2 (0.6)	
B	12 (12.4)	70 (21.4)	
C	84 (86.6)	255 (78)	
MELD, mean ± SD	20.76 ± 7.72	18.61 ± 6.34	0.013
AARC score, median (IQR)	7 (6-8)	9 (9-11)	< 0.001

Variable	Venous ammonia > 152.5 µmol/L, n (%) 99 (23.1)	Venous ammonia ≤ 152.5 µmol/L, n (%) 329 (76.9)	p-Value
ACLF, *n* (%)			
Grade I	47 (47.5)	238 (72.3)	
Grade II	46 (46.5)	87 (26.4)	
Grade III	6 (6.1)	4 (1.2)	
Ascites, *n* (%)	82 (82.8)	308 (93.6)	0.001
Upper GI bleeding, *n* (%)	22 (22.4)	57 (17.3)	0.252
SBP, *n* (%)	4 (4)	20 (6.1)	0.440
Other infections, *n* (%)	16 (16.2)	49 (14.9)	0.758
AKI, *n* (%)	16 (16.2)	32 (9.7)	0.075
HCC, *n* (%)	5 (5.1)	6 (1.8)	0.137
Hospital stay (d), median (IQR)	8 (6-11)	5 (3-10)	0.969
In-hospital death rate, *n* (%)	24 (24.2)	10 (3)	< 0.001

IQR: Interquartile range; SD: Standard deviation; INR: International normalized ratio; WBC: White blood cells; CRP: C-reactive protein; MELD: Model for end-stage liver disease; AARC score: APASL ACLF Research Consortium score; HE: Hepatic encephalopathy; GI: Gastrointestinal; AKI: Acute kidney injury; SBP: Spontaneous bacterial peritonitis; HCC: Hepatocelular carcinoma

Discussion. Our study provided evidence for the utility of VA in predicting the short-term prognosis in patients with ACLF. We found a statistically significant risk for in hospital mortality in patients with high VA that was independent of the disease severity as evaluated by the classic and novel prognostic scores. There is still much controversy regarding the use of VA in patients with LC, concerning its value as a predictor for the development of HE, as well as its role in predicting short-term mortality. VA is considered to be unspecific for the severity of HE (Ong et al., 2003).

However, the utility of VA might reside in its prognostic value. For the vast majority of patients with decompensated LC, VA has not been established as an adequate indicator for poor outcome (Ong et al., 2003). This is mainly due to the intricate mechanisms that lead to hyperammonemia. These mechanisms are found in numerous conditions associated with LC, such as sarcopenia, gastrointestinal bleeding, infection, and AKI, all of which are associated with poor prognostic in patients with LC and ACLF (Patwardhan et al., 2016). There is data suggesting that sepsis and systemic inflammation exacerbate the deleterious effects that ammonia exercise on the brain (Sawhney et al, 2016). Thus, patients with sepsis and LC could present HE even in the absence of high VA. By analyzing only patients with ACLF in our study, we found that elements suggestive of systemic inflammation such as white blood cells and CRP presented higher values in the group of non-survivors, without relation to bacterial infections.

These findings suggest alternative pathways of developing an exacerbated inflammatory response in ACLF, other than through infection, such as endotoxemia which is secondary to bacterial translocation from the gut (Takaya et al., 2020). However, no statistically significant differences were noted in patients with and without HE or high ammonia levels regarding these inflammatory markers. These results suggest that an exacerbated inflammatory response poses a risk for in-hospital mortality in patients with ACLF, mostly determined by other physiopathological mechanisms leading to different organ dysfunctions, such as AKI. The results also indicate that the relationship between systemic inflammation and HE is complex, requiring a more in-depth analysis.

The management of patients with advanced liver disease has been greatly improved by the efforts made to identify the groups with high-risk for mortality, via numerous prognostic scores. This stratification has been of paramount importance for the in hospital management,

facilitating decisions such as admission to the intensive care unit, or urgent liver transplantation (Trebicka et al., 2020).

The traditional prognostic scores (Child-Pugh and MELD) seem to be more adequate for the prediction of outcome in the setting of decompensated LC, but not ACLF (Weil et al., 2017). Moreover, VA showed good accuracy for predicting the outcome, similarly to the accuracy of the MELD score. We identified a cut-off value of 152.5 μmol/L for VA which accurately predicted mortality in our cohort. These observations are in accordance with recent data which supports the theory that ammonia has an independent role in the risk for short-term mortality (Kumar et al., 2012). As discussed, VA levels can be increased in the presence of several conditions frequently associated with LC, among which sarcopenia, AKI, VB, and infection, thus ammonia levels could represent an additional marker of advanced liver disease, indicative of the altered homeostasis of patients with LC and ACLF (Patwardhan et al., 2016). VA could therefore aid in the stratification of ACLF patients regarding in-hospital prognostic and serve as a marker of severity for this category.

In our study, comprising of patients with advanced liver disease, the presence of HE, in general, was not associated with increased mortality. However, grade III or IV HE posed a statistically significant high risk for in-hospital death and was associated with high levels of VA. These results are in accordance with the findings reported by Bajaj et al (Bajaj et al., 2017). The authors analyzed 1560 patients, from which 516 presented HE, 371 grade 1-2 and 145 grade 3-4. Grade 3-4 but not grad 1-2 HE was associated with both higher in-hospital and 30-day mortality rates (Bajaj et al., 2017). Ammonia levels correlate with HE grade in ACLF patients, but high ammonia levels have also been associated with the development of organ failure in the setting of ACLF, other than HE, thus contributing to the unfavorable prognostic of these patients.

Our data suggest that the main risk factor for in-hospital mortality in patients with high VA remains severe HE, thus optimal management of these patients is required.

There are several limitations to our study. As a single-center retrospective study, the possibility of selection bias could not be eliminated. Also, as per hospital protocol, arterial ammonia was not available and therefore not comparable with VA regarding the outcome.

Furthermore, computed tomography cranial scan was not routinely performed for patients with ACLF and HE, thus the frequency and the impact of cerebral edema on survival could not be optimally analyzed.

Conclusions. Our results suggest that VA presents a good predictive value for in-hospital mortality in patients with ACLF and that high levels of VA are associated with severe HE.

VA has the potential to be used as an additional prognostic marker in the evaluation of patients with ACLF. However, prospective additional studies are required to confirm the results.

ACLF is an extraordinary complication of chronic liver disease with a very poor prognosis. Identification of very accurate predictive factors of evolution to these patients could be further used to optimize and intensify treatment patients with acute liver failure and elevated ammonia levels. VA appears to have a good predictive value for in-hospital mortality in patients with ACLF and this will certainly be one of the goals of future research. This topic is a scientific and professional challenge especially as most ACLF patients are assisted in the acute therapy sector where I practice.

Next I will address another complication of LC; portal vein thrombosis is a serious complication of cirrhosis due to further increase in portal venous pressure and decreased blood flow to the liver, with the risk of variceal bleeding and worsening of the liver function (Englesbe et al., 2010; Werner et al., 2013).

2.6. PORTAL VEIN THROMBOSIS AND OTHER THROMBOTIC EVENTS IN PATIENTS WITH LIVER CIRRHOSIS

2.6.1. Introduction

In the last years, the axiom that says that the patient with liver cirrhosis is predisposed to bleeding has proved to be wrong (Northup and Caldwell, 2010).

The liver plays a central role in maintaining the critical balance between bleeding and thrombotic events. LC is characterized by a complex picture of impaired coagulation, thrombocytopenia, decreased pro- and anticoagulant factors produced by the liver, increased von Willebrand factor, factor VIII, and decreased pro- and antifibrinolytic factors, with a low tendency to hyperfibrinolysis (Lisman and Porte, 2010; Tripodi and Mannucci, 2011). Despite clear evidence of an increased tendency for bleeding in patients with liver cirrhosis, in some circumstances these patients are characterized by a hypercoagulable state (Tripodi et al., 2011).

In cirrhotic patients also, the pathogenesis of venous thrombosis is based on Virchow's triad: venous stasis, hypercoagulability, and venous injury.

The accumulation of thrombotic risk factors results in an incidence of deep vein thrombosis in cirrhotic patients ranging from 0.9 to 1.8%, increasing to 8.1% in hospitalized patients (Northup et al., 2006; Garcia-Fuster et al., 2008; Wu and Nguyen, 2012), while the incidence of PVT is 1% in patients with compensated liver cirrhosis, reaching 25% in those on the liver transplant list (Francoz et al., 2012). The incidence of PVT in compensated LC was reported between 0.6% and 5%, and much higher (15%–25%) in decompensated disease (Nonami et al., 1992); Ogren et al., 2006; Tsochatzis et al., 2010). There are no data regarding the difference in the prevalence between partial and total PVT in cirrhotic patients

The impact of PVT on the natural history of cirrhosis remains unclear (Luca et al., 2012; Muruyama et al., 2013). Also, the natural course of PVT in patients with LC is not well known. Moreover, there are many asymptomatic cirrhotic patients in whom PVT is detected incidentally on abdominal ultrasound, and it is not established whether such patients need anticoagulant therapy (Luca et al., 2012). At present, there is neither consensus nor are there guidelines regarding the anticoagulant drugs to be used, duration of treatment, and monitoring methods of cirrhotic patients with PVT.

2.6.2. The risk of thrombotic events in patients with liver cirrhosis

Background & aim. Although the cirrhotic patient is no longer considered "anticoagulated" (Tripodi et al., 2010), his thrombotic risk being similar to that in general population (Guyatt et al, 2012) the American College of Chest Physicians does not specify prophylactic measures and a curative treatment of thrombosis in this population (Guyatt et al., 2012), due to the lack of concrete data on the risk factors associated with thrombosis and the impossibility to assess the risk of bleeding associated with anticoagulant therapy.

The aim of this study was to determine the incidence and risk factors associated with venous thrombosis in hospitalized patients diagnosed with liver cirrhosis.

Material and methods. In the study we enrolled patients admitted to the Institute of Gastroenterology and Hepatology Iasi between January 2010 and December 2011, who were diagnosed with liver cirrhosis. Exclusion criteria were previous diagnosis of hereditary thrombophilia, hepato- cellular carcinoma or other known malignancies and patients on anti-coagulation or antiplatelet therapy.

Thrombotic events, defined as deep vein thrombosis and portal vein thrombosis, were diagnosed clinically and confirmed by venous Doppler ultrasound for deep vein thrombosis and abdominal ultrasonography and contrast computer tomography for PVT.

LC severity was evaluated using the MELD score and Child-Pugh score which is based upon the presence of ascites, hepatic encephalopathy, serum bilirubin, INR and serum albumin levels.

To determine the thrombotic risk factors, the group of patients diagnosed with venous thrombosis was compared with a control group of cirrhotic patients without thrombotic events, randomly selected from all cirrhotic patients admitted during the above mentioned interval.

Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL). For comparing the two groups Chi-square test for categorical variables and t Student test for numeric values were used. The thrombotic risk factors were determined using logistic regression for univariate analysis, and Cox regression analysis method for multivariate analysis.

Results. Out of the 3108 patients included in the study, 31 patients were diagnosed with deep vein thrombosis and 47 patients with PVT, which corresponds to a 2.5% incidence of thrombotic events in patients diagnosed with liver cirrhosis, 0.99% for deep vein thrombosis and 1.51% for PVT.

Deep vein thrombosis was located in the calf in 28 patients, and ileo-femoral in 3 patients; the right leg was the most frequently affected, 58% of cases (18 patients).

In 56% of cases PVT was complete and 5 cases were diagnosed with portal cavernoma. Excluding the 5 cases diagnosed with portal cavernoma, imaging evaluation by Doppler ultrasound and computed tomography could not establish the age of portal vein thrombosis.

None of the patients diagnosed with deep vein thrombosis or PVT had a history of recent surgery (less than one month) or prolonged immobilization.

Out of the 3,030 patients with liver cirrhosis without thrombotic events we have randomly selected a control group of 160 patients in order to establish the risk factors involved in the occurrence of deep vein thrombosis and PVT.

Table 2. XV. Clinical and biological characteristics of the study group

	with DVP / TP n = 78	**without DVP / TP n = 160**	**p-Value**
Age (years)	59.6 ± 11.2	55.2 ± 11.7	0.164
Male gender	49 (62.82%)	101 (63.12%)	0.780
Etiology - toxic	28 (35.9%)	60 (37.5%)	0.083
- viral	33 (42.3%)	66 (41.2%)	0.120
- toxic + viral	15 (19.2%)	29 (18.1%)	0.258
- other	2 (2.6%)	5 (3.2%)	0.138
Ascites	45 (57.69%)	78 (48.7%)	0.075
Hepatic encephalopathy	33 (42.3%)	66 (41.2%)	0.577
Sepsis	17 (21.7%)	11 (6.8%)	< 0.0001
Diabetes mellitus	7 (8.9%)	8 (5%)	0.008
Albumin (mg/dl)	2.5 ± 0.5	3.2 ± 0.7	0.005
Platelets (/mm3)	101.110 ± 51.937	109.100 ± 46.833	0.122
MPV (fl)	10.7 ± 1.27	8.9 ± 0.14	< 0.0001
INR	1.6 ± 0.2	1.5 ± 0.4	0.385
MELD score	13.17 ± 5.39	10.33 ± 4.24	< 0.0001
Child-Pugh score	7.7 ± 1.74	7.9 ± 0.70	0.680

The two groups were comparable in terms of demographic characteristics (age, sex), but the group with thrombotic events was characterized by a higher MELD score ($p < 0.0001$), a higher prevalence of Child- Pugh classes B and C, presence of ascites ($p = 0.083$), hypoalbuminemia ($p = 0.005$), sepsis ($p < 0.0001$), mean platelet volume (MPV) increased ($p < 0.0001$), and diabetes mellitus ($p = 0.008$) (Table 2.XV).

Univariate analysis identified as risk factors for the development of thrombotic events: MELD score>13, albumin <3mg/dl, MVP > 10.5 fl, presence of sepsis and diabetes mellitus

(Table 2.XVi); the remaining clinical and biological parameters did not reach statistical significance.

Table 2.XVI. Univariate analysis of thrombotic risk factors

	OR	95% CI	p-Value
MELD > 13	3.955	1.131 – 15.753	0.009
Albumin < 3mg/dl	3.900	0.981 – 12.182	0.012
MPV > 10.5 fl	2.214	0.269 – 13.850	0.044
Sepsis	2.129	0.755 – 10.468	0.032
DM	3.875	1.278 – 11.336	0.021

Of the above mentioned factors, multivariate analysis has confirmed as independent risk factors of thrombosis in cirrhotic patients: albumin <3 mg/dl, and the presence of sepsis, as a reflection of their advanced liver disease (Table 2.XVII).

Table 2.XVII. Multivariate analysis of rosk factors for venous thrombosis in cirrhotic patients (Cox regression model)

	Coefficient (β)	Wald	HR	95% CI	p-Value
MELD > 13	1.087	12.089	2.94	1.61 – 5.47	0.001
Albumin < 3mg/dl	0.504	5.565	1.65	1.10 – 2.51	0.008
MPV > 10.5 fl	0.301	3.634	1.35	1.00 – 1.84	0.057
Sepsis - Yes	0.262	2.525	1.31	0.94 – 1.80	0.116
DM	0.189	1.498	1.20	0.89 – 1.64	0.221

None of the patients with deep vein thrombosis was diagnosed with pulmonary embolism based on clinical symptoms, but asymptomatic pulmonary embolism has not been evaluated.

Discussion. So far there are several studies that have attempted to assess the thrombotic risk in cirrhotic patients, and some of the results are contradictory. A population-based study conducted in Denmark on 99,000 patients showed that cirrhotic patients have a 1.74 times higher risk of developing deep vein thrombosis than the general population (Sogaard et al., 2009), and the risk was 2.10 times higher in patients aged <55 years. These data, however, were not confirmed by the results of a smaller case-controlled study, carried out by Heit et al, which demonstrated that the association of liver cirrhosis in general population reduced the risk of thrombotic events (RR 0.10) (Heit et al., 2000). The two studies had some limits because they were not aimed primarily to assess thrombotic events in cirrhotic patients, and the data were extracted from a study subanalysis, so that their relevance is low.

Deep vein thrombosis incidence in cirrhotic patients was assessed in eight studies, including two case-control and six retrospective studies, the reported incidence of DVT in cirrhotic patients ranging between 0.5 and 8.1% (Dabbagh et al., 2010; Aldawood et al., 2011). In our study the incidence of deep vein thrombosis was 0.9%, similar to data reported in the literature.

Non-malignant PVT in cirrhotic patients has an incidence of 5-26% (Francoz et al;, 2012). The incidence of 1.51% found in our study was lower than that reported in the literature, but it must be mentioned that previous studies have been conducted in patients on liver transplantation list, diagnosed with advanced liver cirrhosis.

Although surgical interventions and prolonged immobilization are major risk factors for the development deep vein thrombosis in the general population (Guyatt et al., 2012), in our study no patient diagnosed with deep vein thrombosis or PVT had a history of recent surgical intervention or prolonged immobilization, supporting the hypothesis that the risk factors involved in the occurrence of thrombosis in cirrhotic patients are different from the general population.

Our study showed that sepsis is a risk factor for the development of thrombotic events in patients with cirrhosis, confirming the results of the study conducted by Garcia-Fuster et

al., in which 6 of the 17 patients with deep vein thrombosis were diagnosed with sepsis (Garcia-Fuster et al., 2008). Sepsis acts by increasing the direct effect of proinflammatory cytokines, vascular endothelial damage, and also by stimulating platelet aggregation (Vincent et al., 2002). It has been shown that coagulation disorder, defined by elongation of prothrombin time and INR, does not predict the risk of bleeding in cirrhotic patients and does not reflect the true coagulation status in this population; moreover, they do not protect patients from the occurrence of thrombotic events.

In our study, INR values, prothrombin time, as well as platelet count were similar in the two groups. The difference between the two groups occurred when we used mean platelet volume as a method for assessing platelet trombogenity. MPV was higher in patients with thrombotic events, and univariate analysis recognized MPV as a thrombotic risk factor. Mean platelet volume is used to assess the risk for thrombosis in patients with ischemic heart disease (Jaumdally et al., 2007;), without being used, until now, in cirrhotic patients.

Of the biological parameters used to evaluate cirrhotic patients only albumin level <3mg/dl was an independent risk factor for the development of thrombotic events, and reflected poor protein synthesis, including anticoagulant factors (protein C, protein S, antithrombin III).

This is the first national study on the incidence of thrombotic events in patients with cirrhosis and the first in which mean platelet volume as a risk factor for thrombosis in patients with liver cirrhosis was used.

The development of a thrombotic risk scale and a method for the assessment of bleeding risk are essential in deep vein thrombosis and PVT prophylaxis and curative treatment in patients with liver cirrhosis.

Conclusions. The incidence of thrombotic events in patients with cirrhosis was 2.5%, confirming that this population is not "anticoagulated" by definition.

Cirrhotic patients with serum albumin level <3mg/dl and MELD score> 13 have an increased risk for developing thrombotic events, correlated with the severity of liver disease. Thrombosis prophylaxis should be considered in the management of cirrhotic patients and the current guidelines for thromboprophylaxis should also include this special patient category.

2.6.3. Platelets indices in chronic liver disease

Despite thrombocytopenia, platelets are important in LC, playing a major role in hemostasis and other important functions such as inflammation, host defense and angiogenesis and fibrosis (Hugenholtz et al., 2009; Gasparyan et al., 2011).

Platelet indices include MPV, platelet distribution width (PDW), and platelet large cell ratio (P-LCR).

MPV and other parameters including PDW and plateletcrit (PCT) may be used as indica- tors of platelet function and activation in patients with vein thrombosis (Brækken et al., 2009). A high PDW value could suggest a greater production of larger reticulated platelets. PDW directly measures the variability in platelet size and is a marker of platelet activation (Patterson, 1997). A high PDW value could suggest a greater production of larger reticulated platelets. Of all platelet indices, PCT provides more comprehensive data about total platelet mass because it is equivalent to MPV and platelet count (PLT), where PCT=PLTxMPV/107 (Patterson, 1997). Recently it has been demonstrated that MPV is elevated in patients with deep vein thrombosis and significantly higher in patients with non-cirrhotic PVT.

Several studies have evaluated the platelet indices for the assessment of liver fibrosis in patients with chronic hepatitis B virus (Ekiz et al., 2011; Ceylan et al., 2013) and C virus (Purnak et al., 2013) and some of them have reported that MPV and PDW may give information about liver fibrosis severity in HBV and HCV (Ekiz et al., 2011; Ceylan et al., 2013; Purnak et al., 2013); the relationship between these and other platelet indices and liver

fibrosis is still controversial. Purnak et al. demonstrated that increased MPV is an independent predicting factor for liver fibrosis in patients with chronic HCV (Purnak et al., 2013). However, to our knowledge, the association between platelet indices and liver fibrosis in hepatitis C virus related liver disease remains mainly unknown.

In view of these data, we have proposed in these studies to evaluate the influence of platelet indices in patients with PVT and those with HCV.

2.6.3.1. Platelet indices in patients with de novo portal vein thrombosis and liver cirrhosis.

Aim. Therefore, we aimed to investigate whether platelet indices (MPV, PDW, and PCT) could have a diagnostic value for PVT in cirrhotic patients, despite thrombocytopenia and also to evaluate whether platelet indices correlate with hepatic fibrosis measured by TE in patients with chronic HCV.

Study design. *Study population.* It was a case-control study on patients with liver cirrhosis admitted to a tertiary hospital in which a diagnosis of PVT was made during the study interval. Excluded criteria from the study were the patients previously diagnosed with hereditary thrombophilia, hepatocellular carcinoma or other known malignancies, patients on anticoagulant or antiaggregant therapy, and patients with acute or chronic inflammatory diseases, severe anemia, renal failure, acute coronary syndrome, and chronic pulmonary disease.

Portal vein thrombosis diagnosis. PVT was diagnosed by abdominal ultrasound and Doppler ultra- sound after 8 hours fasting. A Philips HD11XE ultrasound machine equipped with a 3.5 MHz convex transducer was used. PVT was confirmed by computed tomography with IV contrast and PVT was classified into 2 categories: complete and partial.

Laboratory data. Samples for complete blood count analysis were obtained by phlebotomy using minimal pressure and collected into a vacutainer tube, containing ethylenediaminetetraacetic acid (EDTA). These blood samples were analyzed within 2 h on automatic blood counter Sysmex XT-4000i (Kobe, Japan). All tests were performed according to the standardized protocols recommended by the manufacturers (Buttarello M and Plebani M. 2008).

For the determination of platelet indices we used an automated hematology analyzer that could overestimate platelet count especially in thrombocytopenic samples compared with the immunological reference method. The platelet indices were derived from the platelet distribution curve obtained from impedance method. It includes MPV that describes the average platelet size reported in femtolitre (fL), PDW defined as the distribution width at 20% frequency level calculated as the standard deviation of platelet volume divided by MPV, and measures the heterogeneity in platelet size. Derived platelet indices are highly specific to the individual technologies, with different analyzers having different reference ranges. As separate feature, the platelet large cell ratio, P-LCR, can be reported specifically on Sysmex analyzers and is the percentage of platelet larger than 12 fL. The reference ranges in our hematology laboratory are: MPV: 8.5 to 12.0 fL, PDW: 10.0 to 18.0 %, and PCT: 0.12 to 0.40 %.

Statistical analysis. Data were analyzed using SPSS Soft- ware Version 17.0 for Windows (SPSS Inc.,Chicago, Illinois). Continuous variables were presented as mean ± standard error and categorical variables as frequency and percentage. Student's t test was used to com- pare normally distributed continuous variables, and the Mann-Whitney U test for variables without normal distribution. The $\chi 2$ test or the Fisher exact test was used to compare categorical variables. Pearson analysis was used to analyze the correlations between platelet indices. Separate logistic regression analyses were used to identify univariate predictors of PVT, and a subsequent stepwise (forward conditional) regression analysis was performed (parameters with a p value of <0.1 in univariate analysis were included in the model). Receiver operating characteristic (ROC) analyses were used to determine the cutoff values

and the sensitivity/ specificity of platelet indices. The odds ratios (OR) and the 95% confidence intervals (CI) were calculated. A 2- tailed p value of <0.05 was considered statistically significant.

Results. There were no significant differences between the two groups in age, sex, clinical risk factors (hypertension, diabetes, smoking), and laboratory parameters (cholesterol, fasting plasma glucose, white blood cell count, hemoglobin, platelet count, etiology of LC, MELD score and Child-Pugh class) (Table 2.XVIII).

Table 2.XVIII. Baseline demographic, clinical and laboratory characteristics of the study population

Characteristics	PVT group (n=54)	Control Group (n=54)	p-Value
Age, y	61.5±11.0	62.3±12.3	0.471
Male,%	34(62.9%)	33(61.1%)	0.755
Diabetes,%	7(12.9%)	8(18.4%)	0.438
Smoking,%	22(40.7%)	18(33.3%)	0.122
Etiology (alcoholic/virus B/C/other)	23/4/25/2	23/6/22/3	0.246
MELD score	12.6±5.5	11.9±7.5	0.685
Score Child	7.6±2.3	7.8±2.1	0.724
Child-Pugh class (A/B/C)	20/10/24	18/14/22	0.111
Blood glucose (mg/dl)	128.9±60.1	137±83.7	0.366
WBC count, per ml	9860±3240	8844±3498	0.229
Hemoglobin, g/dl	12.2±2.07	11.4±2.5	0.584
AP	75.2±19.6	73.7±20.6	0.256
Platelet count, $x10^9$ per L	176±11.2	141±14.3	0.239
Abbreviations: PVT, portal vein thrombosis, WBC, with blood cell, AP prothrombin activity			

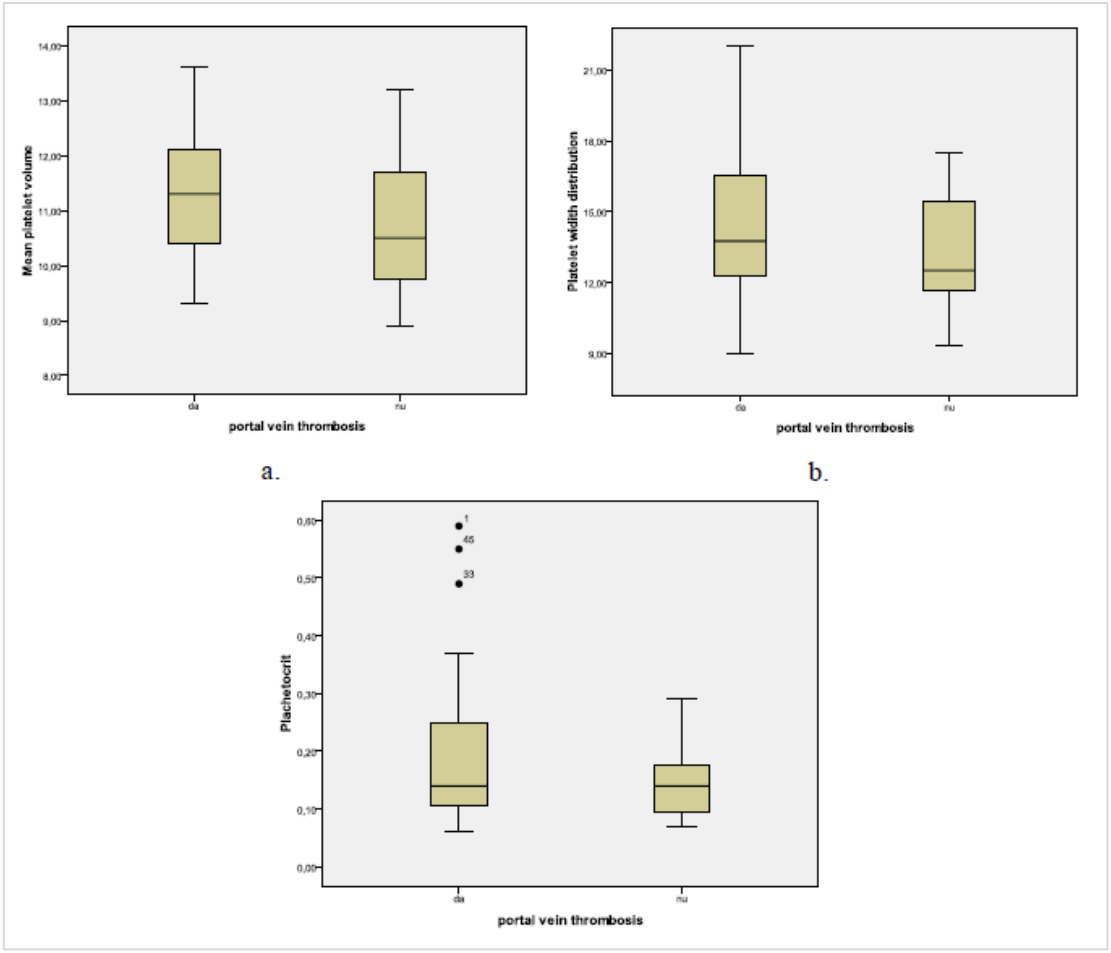


Fig. 2.9. Platelet indices in patients with liver cirrhosis with and without PVT: a-MPV, b- PDW, c- plachetocrit.

According to the time scale of PVT, 25 (46.2%) patients had acute and 29 (53.8%) chronic PVT; PTV was complete in 15 (27.7%) cases and partial in 39 (72.3%). MPV was significantly higher in the PVT group compared with the controls (811.2 + 1.0 fL vs 9.7+1.2 fL [95% CI0.07-0.99], p=0.05). PDW was also significantly higher in the PVT group compared with the control group (14.3 + 3.13 % vs 12.2+2.38% [95% CI 0.10-2.48], p=0.048, respectively). PCT was, however, significantly lower in PVT group compared with the controls (0.19+0.12% vs 0.14+0.06% [95% CI 0.08-0.09], p=0.015, respectively). In addition, there was a significant inverse relationship (Figure 2.9).

To determine the best cutoff values of platelet indices for predicting PVT, ROC analyses were performed. The areas under the ROC curves for these indices used to detect PVT were calculated (Table 2.XIX) and the sensitivity and specificity of the best cutoff values were determined (Table 2.XX).

Table 2.XIX. Receiver operating characteristics analysis for platelet indices to predict PVT

Variable	β	±SE	p-Value
MPV	-0.053	0.740	0.046
PDW	0.011	0.258	0.012
PCT	-7.584	5.972	0.026

Table 2.XX. Sensitivity and specificity of the cutoff values of plateles indices

Variable	Area under the ROC curve	95% CI	p-Value
MPV	0.610	0.46-0.75	0.022
PDW	0.579	0.43-0.71	0.035
PCT	0.576	0.44-0.71	0.038

Patients with PVT and liver cirrhosis had abnormalities of platelet activation compared with the group without PVT, and these platelet abnormalities may partly contribute to the pathophysiology of PVT. Platelet activity indices including MPV, PDW, and PCT may be used in predicting PVT in patients with liver cirrhosis.

Discussion. In the present study, we have found that the platelet activity indices were significantly and independently associated with the presence of PVT. Platelet activity can be influenced by a multitude of variables, therefore it is difficult to predict the extent of platelet reactivity in an individual. A practical, reliable, and available index of platelet activation has not been discovered although considerable amount of research has been performed (Patterson, 1997).

Various laboratory methods including platelet count and size, aggregates, and released substances from activated platelets have been introduced for the detection of platelet activity (Patterson, 1997). However, they have different advantages and disadvantages. MPV is an inexpensive and easily available biomarker. It is routinely available in inpatient and outpatient setting at a relatively low-cost. Increased MPV values are related to various cardiovascular risk factors, disorders and inflammatory processes resulted in arterial and venous thromboses. Circulating platelets are heterogeneous in size, metabolism, functional activity, and density. Larger platelets are metabolically and enzymatically more active than smaller platelets, containing more prothrombotic material, with increased thromboxane A2 and B2 per unit volume and glycoprotein IIb-IIIa receptor expression (Giles et al., 1994). In healthy participants, there is a nonlinear inverse correlation between MPV and platelet concentration: MPV tends to decrease in participants with higher platelet counts, as found in our study (Giles et al., 1994).

In previous studies, increased MPV was found to be associated with non-alcoholic fatty liver disease, hepatitis C and B and advanced fibrosis (Bowles et al., 2005). Braekkan et al.

reported that MPV is a risk factor in venous thromboembolism (Braekkan et al., 2009). Our findings provide further evidence that platelet activation, measured by elevated MVP, PDW and PCT may contribute to the pathogenesis of venous thrombosis.

Pizzuli et al. suggested that because platelets stay in the circulation 7-11 days, they might be detected days before symptoms appear (Pizzuli et al., 1998). We believe that the same correlation might exist between platelet indices and PVT. At routine checkup a cirrhotic patient with elevated MPV, PCT and PDW might be a suspect for PVT, despite thrombocytopenia. However, to demonstrate the possible predictive value of platelet indices for PVT more prospective studies on larger patient groups should be performed.

Our data suggested that increased platelet indices contribute to the prethrombotic state in liver cirrhosis and that larger platelets may play a specific role in thrombosis despite thrombocytopenia. Platelet indices could provide useful clinical information and be built into a risk assessment algorithm for PVT. This study supports the fact that platelet reactivity is important in the pathogenesis of PVT. An important limitation concerning the predictive value of MPV, MPM, and MPC is the absence of well-defined limits to differentiate between activated and nonactivated platelets. Other platelet activity indices, such as radio-labeling methods, aggregometry procedures, platelet-specific eicosanoids, other release reactions such as serotonin and histamine, adhesion molecules, flow cytometric investigations, platelet function analyzers, thromboelastography, and plasma factors influencing platelet activation such as fibrinogen were not used in our study.

To our knowledge, this study has examined for the first time the relationship between all platelet indices, PVT and liver cirrhosis

2.6.3.2. Platelet indices and liver fibrosis evaluation in chronic hepatitis C

Study design. *Study population.* It was a prospectively study that included patients with known chronic HCV naïve to antiviral treatment and followed-up in a tertiary care center, who have TE examination in order to evaluate platelet indices correlate with hepatic fibrosis.

Exclusion criteria involved all conditions that are known to modify platelet indices such as chronic liver diseases other than HCV, type 2 diabetes mellitus, hypertension, hypercholesterolemia, obesity, malignancy, and hypochromic anemia (Buttarello and Plebani, 2008; Leader et al., 2012;). None of the patients included have received anticoagulant or antiaggregant medications, non-steroidal anti-inflammatory drugs and oral contraceptives before hospital admission because all these drugs could influence platelet aggregability.

Laboratory data, serum measurements /statistical data – all data are similar to the previous study, so we do not repeated.

Liver fibrosis assessment. All patients had liver stiffness measured by TE (FibroScan®, Echosens, Paris, France). At least 10 valid measurements, a 60% success rate, and an interquartile range of less than 30% of the median elasticity were required for a measurement to be valid. The liver stiffness was expressed in kiloPascal (kPa), and values range from 2.5 to 75 kPa, with normal values <5.5 kPa (18). According to Metavir score, a cutoff value of 8.7 kPa defines mild (5.6-7.1 kPa=F1) or moderate (7.2-8.7 kPa=F2) fibrosis, while values ≥ 8.7 kPa defines advanced fibrosis (8.8-12.5 kPa=F3; ≥12.5 kPa=F4). In this study patients were divided in two groups: mild/moderate fibrosis (F1-F2) and advanced fibrosis (F3-F4).

Results. There was a significant difference regarding the MVP, PDW and P-LCR values between the patients with mild/moderate fibrosis and those with severe fibrosis (P=0.002, P=0.003, P<0.0001). Platelets counts were significantly lower in patients with advanced fibrosis compared with those with mild/moderate fibrosis (240.8±52.1 vs. 156.7±75.8, P<0.0001) (Table 2.XXI).

Table 2.XXI. Platelet indices in relation to the fibrosis stages in HCV patients

Variables	F1-F2 median (IQR) (n = 64)	F3-F4 median (IQR) (n = 75)	F	p-Value
MPV, fL	10.5 (0.90)	11.0 (1.7)	9.820	0.002
P-LCR, %	28.8 (6.70)	32.5 (13.90)	13.762	<0.0001
PDW, %	12.65 (2.10)	13.9 (4.75)	9.362	0.003

Our study showed that high platelet indices (MPV, PDW and P-LCR) could differentiate between mild/moderate and advanced fibrosis in HCV patients. These results confirmed some previously mentioned studies including patients with chronic HCV (Purnak et al., 2013).

Discussions. Transient elastography (Fibroscan®) is reproductible, non-invasive and validated test for the assessment of liver fibrosis (Ekiz et al., 2011). Both Fibroscan® and some patent-protected serum markers for liver fibrosis are still expensive. It was this context that assessment of platelet indices triggered much interest as possible non-invasive, widely available, practically costless serum markers of liver fibrosis in patients with chronic HBV or HCV (Ekiz et al., 2011; Ceylan et al., 2013; Purnak et al., 2013). Some studies have suggested that elevated MPV levels may be an independent predictor of cirrhosis (Qi et al., 2014) or associated with liver disease activity (Hu et al., 2014) in patients with chronic HBV or portal vein thrombosis (Girleanu et al., 2013).

Purnak et al. (Purnak et al., 2013) demonstrated that MPV is increased in patients with advanced fibrosis, and this marker along with other serological markers could give information about liver fibrosis severity in chronic HCV. The authors demonstrated that patients with HCV had significantly higher levels of MPV as compared to the control group, despite the same number of platelets, concluding that high levels of MPV could represent an indirect early marker of advanced liver fibrosis in such patients.

We found that high platelet indices (MPV, PDW and P-LCR) could differentiate between mild/moderate and advanced fibrosis in HCV patients. It has been reported that increased platelet indices are directly related with thrombocytopenia, which is a direct reflection of platelet destruction by hypersplenism (Lisman and Porte, 2012). In advanced stages of fibrosis thrombocytopenia is a multifactorial condition attributed to the sequestration of platelets by the spleen secondary to portal hypertension, reduced thrombopoetin which regulates megakaryocyte maturation and platelet production, and bone marrow suppression (Lisman and Porte, 2012). All these mechanisms could influence platelet indices, because thrombocytopenia is not only a reflection of platelet destruction, but also of platelet production, especially in advanced chronic liver disease.

Conclusions. Our results suggest that increased platelet indices contribute to the prethrombotic state in liver cirrhosis and that larger platelets may play a specific role in thrombosis despite thrombocytopenia.

Furthermore, we showed that the platelet indices, MPV, PDW and P-LCR are associated with fibrosis stage measured by TE in patients with chronic HCV, and they might be used as markers to evaluate liver fibrosis stage. This study has some advantages: such as being the first prospective study that evaluated the role of all platelet indices in patients with chronic HCV, using TE for liver fibrosis quantification. The study has also some limitations as relatively small sample size, and lack of evaluation of dynamics of platelet indices. Our results need to be confirmed by further investigations on larger groups of patients.

2.6.4. Natural Course of Nonmalignant Partial Portal Vein Thrombosis in Cirrhotic Patients

Background & aim. The define the natural course of PVT in LC is mandatory to establish the indication of anticoagulant treatment and to evaluate its efficacy. Several studies have reported that spontaneous recanalization of the portal vein in the absence of any specific therapy is unusual, especially in total PVT (Francoz et al., 2005; Amitrano et al., 2010; Delgado et al., 2012) but the results are controversial, depending on the study design (Senzolo et al., 2012; Werner et al., 2013;). Subsequently, there are some unanswered questions regarding PVT and the progression of LC, the impact on LC natural history, or the rate of spontaneous recanalization.

The aim of this study was to evaluate the natural history of nonmalign partial portal vein thrombosis and its impact on the long-term outcomes in cirrhotic patients.

Patients and methods. *Study population.* We conducted a prospective cohort study on cirrhotic patients admitted in a tertiary referral center. Patients diagnosed with partial PVT between January 1, 2011, and December 31, 2011, were followed up until October 30, 2013, or death. We excluded patients who received anticoagulant treatment, patients with malignant disease including hepatocellular carcinoma, known thrombofilia, patients with a history of transjugular intrahepatic portosystemic shunt and those with portal cavernoma.

A written consent was obtained from all the patients. The study was performed in accordance with the Declaration of Helsinki and approved by our local Ethics Committee.

Data collection. From each patient we collected the following information: age, gender, international normalized ratio, serum bilirubin and albumin, platelet counts, and etiology of LC. The diagnosis of liver cirrhosis was established based on clinical manifestations and biological, endoscopic, and ultrasound changes suggestive for advanced liver disease and portal hypertension. LC severity was evaluated using the MELD score and Child–Pugh class.

Patency of the portal vein was assessed by abdominal ultrasonography and Doppler ultrasonography in all screened patients at the time of enrollment. All patients diagnosed with PVT based on Doppler ultrasonography had contrast-enhanced computed tomography to confirm the presence and extension of PVT. Partial PVT was defined as the presence of a hyperechogenic material in portal lumen without complete obstruction.

Events definition. PVT was considered improved when complete recanalization or a reduction of more than 50% of the thrombus was achieved, stable when the thrombus maintained the same dimensions or there was a reduction less than 50%, and worsened when the thrombus was extended to superior mesenteric vein, splenic vein, or complete PVT (Senzolo et al, 2012).The patients were evaluated every 3 months by abdominal ultrasound combined with Doppler examination, and at 6 months by computed tomography, for a mean observational period of 20.22 ± 8.6 months.

Statistical analysis. Data were analyzed with the SPSS Software Version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean ± standard deviation and categorical variables as frequency and percentage. Student's *t* test was used to compare normally distributed continuous variables and the Mann–Whitney *U* test for variables without normal distribution. The λ^2 test was used to compare categorical variables. Kaplan–Meier method was used to determine if there was a significant difference in clinical events between the study subgroups. Independent predictors for survival and decompensation were assessed by using a Cox proportional hazards model. A two-tailed *P* value < 0.05 was considered as statistically significant.

Results. *General characteristics.* There were 1580 patients with cirrhosis over the screening interval, out of them 121 were associated PVT. Among cirrhotic patients with PVT, 99 patients were considered to be not eligible according to the inclusion/exclusion criteria (79 with concomitant/history of hepatocellular carcinoma, 4 with insufficient laboratory data, 12

with other malignant diseases, 3 with anticoagulant treatment for cardiac diseases, and 1 with transjugular intrahepatic portosystemic shunt). Thus, the study included 22 patients (12 males, 10 females, mean age 61.45 ± 9.63 years; range, 29–80 years). Chronic viral hepatitis was the main cause of LC in half of the patients. Nine patients (40.9%) were asymptomatic, 10 patients (45.5%) were admitted with abdominal pain, and in 3 patients (13.6%) PVT diagnosis was associated with variceal bleeding. The majority of the patients had thrombosis of a single vessel (81.1%) and PVT involved the right portal vein in 3 patients and left portal vein in 4 patients. The overall mean observation period was 20.22 ± 8.61 months, ranging from 4 to 31 months. Baseline characteristics of the cirrhotic patients with partial PVT are summarized in Table 2.XXII.

Course of partial PVT. During the follow-up period, PVT remained stable in 11 (50%) patients, improved in 5 (22.73%), and worsened in 6 (27.27%) patients. In 2 patients complete portal vein recanalization was obtained after a mean follow-up period of 10.5 months. At the time of enrollment, there were 4 patients with PVT extension to superior mesenteric vein (one of them with splenic vein involvement), all remaining stable during follow-up. None of the patients received anticoagulant treatment. The patients (n = 18) with thrombus limited to portal vein had not developed extension to superior mesenteric vein or splenic vein during the study period.

Table 2.XXII. Baseline characteristics of the patients with LC and partial PVT

Characteristics	PVT (n=22)
Age, years (mean ± SD)	61.45 ± 9.63
Male *n*, %	12 (54.54%)
Diabetes *n*, %	3 (13.63%)
Smoking *n*, %	10 (45.45%)
Etiology (alcoholic/viral/other)	6/11/5
MELD score (mean ± SD)	12.73 ± 4.34
Child-Pugh score (mean ± SD)	7.7 ± 1.82
Child-Pugh class (A/B/C)	7/9/6
Blood glucose, mg/dL (mean ± SD)	117.08 ± 46.79
WBC count, per mL (mean ± SD)	6129.54 ± 2552.44
Hemoglobin, g/dl (mean ± SD)	11.26 ± 1.85
INR, % (mean ± SD)	1.23 ± 0.16
Platelet count, ×109 per L (mean ± SD)	112 ± 74.86
Ascites	
Absent	10
Mild	3
Moderate	8
Severe	1
Esophageal varices	
Small	2
Medium	6
Large	14
Vessels with thrombosis	
PVT	18
PVT, SMV	3
PVT, SMV, and splenic vein	1

MELD: Model of end-life disease, SD: Standard deviation, WBC: White blood count, INR: International normalized ratio, PVT: Portal vein thrombosis, SMV: Superior mesenteric vein

Clinical outcomes. The correlation between the natural course of PVT and clinical evolution is summarized in Table XXIII.

Table XXIII. Correlation between the natural course of partial vein thrombosis and clinical evolution

Parameter	Stable / improved (n = 16) (%)	Worsened (n = 6) (%)	p-Value
Esophageal varices (size)			
Small	2 (12.5)	1 (16.6)	0.230
Medium	8 (50.0)	1 (16.6)	<0.0001
Large	6 (37.5)	4 (66.6)	<0.0001
Variceal bleeding	5 (31.2)	5 (83.3)	<0.0001
Refractory ascites	6 (37.5)	4 (66.6)	<0.0001

The Kaplan–Meier probability of episodic hepatic decompensation at 6 and 18 months was 0.95 [95%confidence interval (CI) 0.41–0.99], and 0.70 (95% CI 0.05–0.80) (Figure 2.10). The rate of 6 months decompensation was 31.8%, and 68.1% at 18 months. There was a clear association between progression or regression of partial PVT and clinical outcome ($\lambda 2$ = 27.677, P < 0.0001) (Figure 2.11).

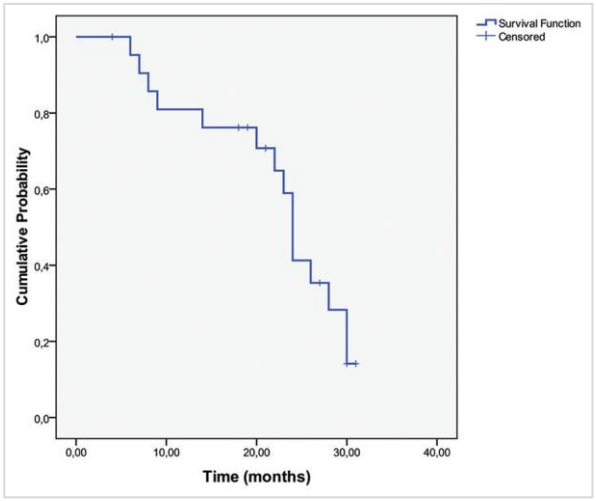


Figure 2.10. Kaplan–Meier plot showing the probability of patients with PVT remaining without decompensation over the follow-up period

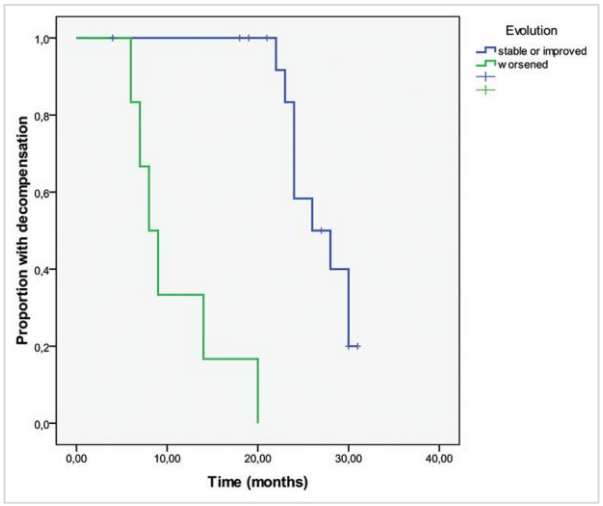


Figure 2.11. Kaplan–Meier plots showing the probability to remain without decompensation in patients with stable/improved PVT compared with those with worsened partial PVT

Eleven (68.7%) patients with stable/improved PVT and three (50%) of those with worsened PVT remained free of decompensation at 6 months (P = 0.006). At 18 months, seven (43.7%) patients from those with stable/improved PVT and none of the patients with worsened PVT remained free of decompensation (P < 0.0001).

The Kaplan–Meier probability of survival at 6 and 18 months was 0.95 (95% CI 0.52–0.99), and 0.74 (95% CI 0.21–0.78) (Figure 2.12). There was a clear association between progression or regression of partial PVT and survival ($\lambda 2$= 6.347, P < 0.0001) (Figure 2.13).

The rate of survival at 6 and 18 months in the first group (PVT stable/improved) was higher compared with the second group (worsened PVT) (81.2% vs. 66.6%, P = 0.005; 81.2% vs. 16.6%, P < 0.0001). At the end of the study, the mortality rate was 56.2% in the first group and 100% in the second group of patients (P < 0.0001). Medium survival time was 19.22 months in the first group and 8.6 months in the second group of patients (P < 0.0001).

Multivariate analysis showed that the MELD score at diagnosis of PVT in cirrhotic patients was the only independent predictor of survival ([hazard ratio (HR) 1.76; 95% CI: 1.06–2.92, P = 0.028) and hepatic decompensation (HR 1.42; 95% CI: 1.08–1.87, P = 0.012).

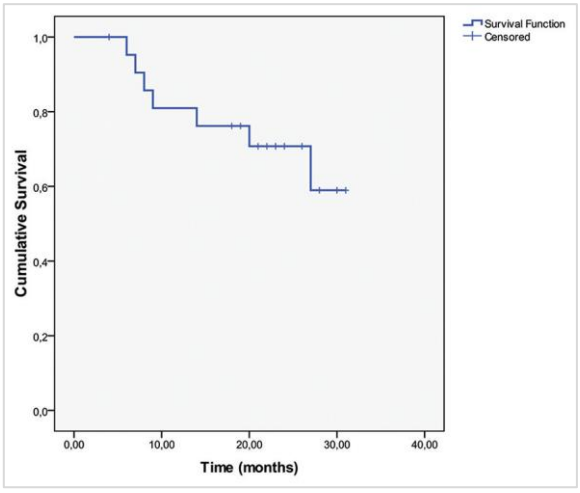


Figure 2.12. Kaplan–Meier plot showing the probability of survival in patients with partial PVT over the follow-up period

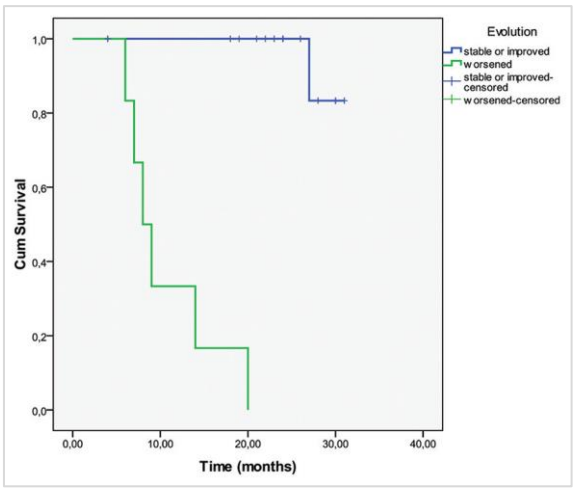


Figure 2.13. Kaplan–Meier plots showing the survival probability of patients with stable/improved partial PVT compared with those with worsened partial PVT

Discussion. To date insufficient data are available on the natural evolution of PVT (Luca et al., 2012; Muruyama et al., 2013). The aim of this study was to establish the natural history of nonmalignant partial PVT and the influence of PVT on the outcomes in patients with cirrhosis, in order to identify subgroups of patients who could benefit from PVT treatment. Our study found that more than half of cirrhotic patients diagnosed with partial PVT improved PVT negatively influenced the patient's clinical outcomes. The findings provided evidence that anticoagulant treatment may not be necessary for the majority of cirrhotic patients with partial PVT.

The published data on the natural course of partial PVT are few and contradictory (Yerdel et al., 2000; Francoz et al., 2005; Senzolo et al., 2009; Northup and Intagliata, 2011; Luca et al., 2012; Muruyama et al., 2013). Several studies reported that spontaneous recanalization of PVT is rare (Amitrano et al., 2010; Senzolo et al., 2012; Delgado et al., 2012;). In the study by Francoz et al (Francoz et al., 2005), no patient achieved recanalization of partial and total PVT in the absence of anticoagulation, whereas 42% achieved recanalization on anticoagulant therapy Senzolo et al. (Senzolo et al., 2012) reported thrombus progression in 75% patients who did not receive anticoagulation treatment, compared with only 15% of treated patients. However, Maruyama et al. reported spontaneous improvement in 47.6%, unchanged appearance in 45.2%, and progression in only 7.2%, and found no difference in the natural course of PVT based on the degree of obstruction or the location of the thrombus (Muruyama et al., 2013). In another study aimed to define the natural course of nonmalignant partial PVT in cirrhotic patients, Luca et al. confirmed that in 45% patients partial PVT improved, and thrombus progression did not influence patients' clinical outcome (Luca et al., 2012).

The difference between these studies could be explained at least partially by their design, the primary outcome, small sample size, and short-term follow-up. Definitive diagnosis of PVT can be obtained by computed tomography and magnetic resonance imaging, both methods providing information about the extent of the thrombosis and the development of collateral circulation. Previously, data regarding natural history of partial PVT were extracted from studies evaluating the efficacy of anticoagulant treatment in cirrhotic patients. All these studies (Yerdel et al., 2000; Francoz et al., 2005; Werner et al., 2013; Muruyama et al., 2013) included a heterogeneous population, mostly formed by partial PVT, but with no consensus regarding partial/total PVT definition, which may explain the large range in prevalence of PVT in cirrhotic patients.

Our study confirmed the findings reported by Luca et al. (Luca et al., 2012) although we found that worsened PVT was associated with patients' poor clinical outcome, including mortality.

Our study has some strengths: It is a prospective cohort study, excluded patients with hepatocellular carcinoma where the incidence of PVT is higher and the mechanism of thrombus formation is different (invasive of portal vein by hepatoma cells in addition to abnormalities of coagulation and fibrinolysis systems), (Ponziani et al., 2010) and all patients had imaging evaluation at the time of screening and every 3–6 months in the follow-up period. However, this study has also several limitations; small number of patients included, a single center study, and absence of routine testing for a hypercoagulable state.

Finally, as our study shows that worsened partial PVT has a prognostic value, this variable may be included in the future studies aimed to identify predictor factors of mortality in cirrhotic patients.

Conclusion. Our study shows that more than half of cirrhotic patients with partial PVT had a stable or improved thrombus evolution without anticoagulant therapy, although worsened PVT negatively influenced outcomes. Prospective randomized controlled clinical trials are needed, but until then clinicians should carefully consider the risk of anticoagulant treatment in cirrhotic patients with partial PVT.

The results of this study are similar to those of a previous study in which we followed patients with non-malignant partial PVT for 2 years. Without anticoagulant treatment, we found that thrombosis worsened in 45.4% of cases, improved in 27.27% of patients and remained stationary in 27.27%. Multivariate analyses showed that the MELD score at diagnosis was the only independent predictor of survival and hepatic decompensation.

The last topic I will refer to in this chapter, but not the least important, is HCC, a common complication in patients with chronic liver disease, most often with a poor prognosis. Our primary research topics addressed the particularities of HCC after direct-acting antiviral HCV therapy.

2.7. HEPATOCELLULAR CARCINOMA IN PATIENTS WITH CHRONIC LIVER DISEASE

2.7.1. Introduction

HCC represents the most frequent histologic type among primary liver neoplasia. HCC is the sixth most frequent malignancy and the third leading cause of neoplasia-related death in 2020 worldwide (Bray et al., 2018). The incidence of HCC has been rising worldwide over the last 20 years and is expected to increase until 2030 in some countries.

The incidence of HCC is highest in Asia and Africa, where the endemic high prevalence of hepatitis B and hepatitis C strongly predisposes to the development of chronic liver disease and subsequent development of HCC.

Traditional liver cirrhosis is the main risk factor for HCC but it is not a mandatory condition; ultrasound often shows a suspicious image of HCC even in non-cirrhotic patients.

The leading risk factor for HCC is HCV infection with a 3% annual risk in patients with HCV liver cirrhosis (El-Serag, 2011). Current international vaccination strategies for HBV, and advances in the management of HCV, promise to have a major impact on the incidence of HCC, but their benefit will be realized slowly because of the very long latency period—20-30 years—from hepatic damage to HCC development.

In the last years there is a growing problem with NAFLD and NASH that can progress to cirrhosis and liver failure in 3%-15% of cases. There is a lack of large population studies

regarding the risk of HCC in patients with liver cirrhosis secondary to NASH (Hashimoto et al., 2009).

Accordingly, the identification and management of the modifiable major risk factors such as HBV or HCV, excessive alcohol consumption, aflatoxin, type 2 diabetes mellitus, obesity, and smoking are of essence to interrupt the upward dynamics of the HCC rates (Sung et al., 2021).

In the last 10 years the therapeutic management of HCC considerably improved leading to extended access to therapeutic options that increase the life expectancy of these patients. However, HCC still remains an aggressive malignant tumor with a high rate of cumulative recurrence over 5 years (70%) after curative therapies (El-Serag, 2011).

2.7.2. Long-term Risk of Hepatocellular Carcinoma Following Direct-Acting Antiviral Therapy in Compensated Liver Cirrhosis Induced by Hepatitis C Virus Infection

Background & aim. The carcinogenic potential of HCV has been extensively studied. According to previous studies, its prooncogenic effects on the infected cells are caused by both direct (i.e., DNA damage, oxidative mechanisms) and indirect (i.e., liver injury and fibrosis) mechanisms. Data from the interferon era showed that achieving SVR significantly reduced the risk of HCC, and this finding was a significant contribution to hepatologists (Janjua et al., 2017; Brown, 2000).

According to the beneficial effects of SVR on the HCC occurrence reported after interferon therapy, clinicians anticipated even further benefits, including a significant decrease in the HCC occurrence/recurrence, in cirrhotic patients treated with DAAs. In 2016, shadows were cast over fantastic SVR in HCV patients when two articles from Spain and Italy reported that the DAAs therapy might favor the HCC occurrence or recurrence (Reig et al., 2016; Conti et al., 2016).

While evidence suggests that the risk of HCC does not completely disappear after SVR, it is of great importance to determine the role of DAAs therapy in hepatocarcinogenesis to show whether it is suppressing or promoting the development of HCC. As much data as possible on this topic is immediately required to better manage HCC surveillance, especially for cirrhotic patients.

Our study aimed to determine the long-term risk of de novo HCC and risk factors in patients with HCV genotype 1b compensated cirrhosis, following the achievement of SVR by DAAs regimens. As a secondary objective, this study also aimed to assess the tumor aggressiveness and its impact on treatment decisions.

Materials and methods. *Patients.* This multicentric cohort study analyzed the data from 479 consecutive patients with chronic HCV genotype 1b infection and compensated liver cirrhosis, treatment-experienced or naïve, treated with PrOD +/- RBV for 12 weeks in two tertiary centers in Northeastern Romania. The patients were prospectively followed up from November 2015 to December 2020. The patients were included in the study according to the Romanian National Health Insurance House's criteria: (1) patients with HCV genotype 1b infection; and (2) compensated cirrhosis defined as F4 by transient elastography (> 13 kPa). We excluded patients aged below 18 years at the initiation of treatment and those with concomitant human immunodeficiency virus or hepatitis B virus infection, heavy alcohol intake, and documented malignant neoplastic disease, including HCC.

Monitoring during and after DAAs treatment. The diagnosis of HCV infection was decided based on the serum HCV RNA levels, measured with the COBAS Taq-Man HCV quantitative test (Roche Molecular Systems, Inc.Branchburg, NJ) with a lower limit of quantification and detection of 15 IU/mL. The Child–Pugh and MELD scores were calculated at the baseline, end of treatment, and12 weeks after the therapy. We used transient

elastography (FibroScan; Echosens, Paris, France) to perform the liver stiffness measurement (LSM) with 13 kPa as the cutoff point for cirrhosis.

HCC Surveillance. The HCC screening was performed at the baseline in all patients using abdominal ultrasonography, computed tomography, or magnetic resonance imaging. Abdominal ultrasonography and AFP were performed every 3 - 6 months for all patients after the initiation of treatment.

Ethical Considerations. This study was approved by the National Ethics Committee, and written informed consent was obtained from all participants, while observing the principles of the Declaration of Helsinki.

Statistical Analysis. All data were statistically analyzed using SPSS software version 22.0 (IBM SPSS Inc., Chicago, IL, USA). The continuous variables were expressed as median (first-third quartiles) and compared using Student's t-test; however, the categorical variables were reported as frequencies and percentages and compared using chi-squared or Fisher's exact tests. The Kaplan-Meier method was used to calculate and plot the cumulative HCC incidence. Only complete data were analyzed in this study. To compare the differences among the groups, the log-rank test was used. In this regard, a Cox proportional hazard model with a hazard ratio (HR) and 95% confidence interval generated by Cox regression was calculated in both univariate and multivariate analysis to detect the risk factors associated with the HCC occurrence. Moreover, we evaluated the relationship between AFP and the HCC occurrence using the area under the receiver operating characteristic curve analysis, and the optimal cutoff value was selected at the highest specificity and sensitivity from the receiver operating characteristic (ROC). Statistically, two-tailed $P < 0.05$ was set as the significance level in this study. Kolmogorov-Smirnov test was performed to check the normality of the data distributions.

Results. *Participants.* The study included 479 patients treated with PrOD ±RBV, with a median age of 60 (52-73) years, who mainly encompassed female patients (54.5 %). Of the research participants, 32% had a history of antiviral treatment with interferon, and 16.5% received RBV associated with PrOD. The participants' BMI was 27.76±4.04 kg/m^2 and more than half of the patients (57.2%) had comorbidities. Table 2.XXIV shows the demographic, clinical, biological, and imaging data of the study participants.

Table 2.XXIV. Demographic characteristics of patients at baseline

Parameters	**Values**[a]
Age (y), median (IQR)	60 (52 - 73)
Gender, male/female	218/261 (45.5/54.5)
BMI (kg/m^2), mean ± SD	27.76 ± 4.04
Comorbidities	274 (57.2)
Obesity	16 (3.3)
Hypertension	175 (36.5)
Diabetes mellitus	67 (14)
Personal history of neoplasia	16 (3.3)
IFN experienced	153 (31.9)
Ribavirin	79 (16.5)
Esofageal varices	133 (27.8)
Small	69 (51.8)
Medium	21 (15.7)
Large	43 (32.3)
Child-Pugh score	
5	424 (88.5)
6	55 (11.5)

Abbreviations: BMI, body mass index; IFN, interferon.
[a]Values are expressed as No.(%) unless otherwise expressed

All patients had compensated liver cirrhosis (according to Child Pugh score, 88.5% had a score of 5, and 11.5% had a score of 6).

Direct-Acting Antiviral Therapy Outcomes. All patients completed the 12-week treatment course and achieved SVR after the DAAs therapy. The mean follow-up period was 60.11 3.87 months. A statistically significant decrease was recorded at the ALT (alanine aminotransferase), AST (aspartate aminotransferase), GGT (gamma-glutamyl transferase), and AFP levels and LSM ($P < 0.001$) in SVR compared to baseline. Table 2. XXV represents further information in this regard.

Table 2.XXV. Effect of DAAs on Liver Function

Parameters	Baseline	SVR	p-Value
Platelets (/mm^3)	138 (101 - 181)	146 (105 - 193)	< 0.001
ALT (IU/L)	87 (60 - 123)	24 (19 - 33)	< 0.001
AST (IU/L)	83 (56 - 128)	22 (19 - 30)	0.001
ALP (IU/L)	94 (76 - 116)	92 (73 - 137)	< 0.001
GGT (IU/L)	69 (43 - 110.5)	32 (22 - 46)	< 0.001
Bilirubin (mg/dL)	0.92 (0.68 - 1.27)	0.74 (0.42 - 1.05)	0.233
Direct bilirubin (mg/dL)	0.43 (0.33 - 0.59)	0.43 (0.28 - 0.7)	< 0.001
Albumin (g/dL)	4.04 (3.73 - 4.38)	4.29 (3.94 - 4.6)	0.943
Cholesterol (mg/dL)	160 (146 - 192)	183 (152 - 210.25)	0.039
Triglycerides (mg/dL)	107 (83 - 125)	99.5 (77 - 125.75)	0.545
AFP (ng/mL)	9.28 (5.2 - 16.92)	3.89 (2.76 - 6.75)	0.002
LSM (kPa)	21.55 (16.78 - 32.63)	10.6 (7.1 - 15.3)	< 0.001
Child-Pugh score	5	5	0.235
MELD	8.1 (7 - 10)	7.5 (7 - 8)	0.321

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; AFP, alpha-fetoprotein; ALP, alkaline phosphatase; LSM, liver stiffness measurements; MELD, model for end-stage liver disease

Incidence and Risk Factors of HCC Occurrence. After the mean follow-up of 60.11±3.87 months, 23 patients (4.8%) developed HCC. The 1-, 3-, and 5-year cumulative incidence rates of HCC were 1.1, 1.9, and 2.6%, respectively. The mean period from the beginning of the treatment to the HCC diagnosis was 19.6±13.7 months. All patients in our cohort study had compensated liver cirrhosis (Child-Pugh A 5/6). Baseline factors contributing to the HCC occurrence following DAAs were then assessed (Table 2.XXVI).

Table 2.XXVI. Baseline factors with possible impact on HCC occurrence

Variables	HCC = 23	No HCC = 456	p-Value
Age	63.04 ± 10.1	59 ± 8.14	0.022
Gender, male/female, (male%)	13/10 (57)	251/205 (55)	0.448
PLT, × $10^2/\mu$L	12.6 ± 50.4	14.8 ± 63.9	0.109
BMI	26.9 ± 3.7	27.7 ± 4.04	0.156
AFP baseline	15.8 ± 23.5	13.9 ± 9.7	0.696
ActiTest	0.76 ± 0.13	0.69 ± 0.18	0.037
ALT, U/L	112.78 ± 69.07	103.54 ± 71.56	0.545
AST, U/L	110.87 ± 51.68	101.25 ± 66.88	0.497
GGT, U/L	89.68 ± 76.42	93.98 ± 81.67	0.809
ALP, U/L	113.95 ± 40.73	98.95 ± 33.91	0.046
Treatment experienced, Yes (%)	205 (45)	13 (56.5)	0.312

Abbreviations: PLT, platelets; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; AFP, alpha-fetoprotein; ALP, alkaline phosphatase

The patients who were diagnosed with HCC (n = 23) were older (63 *vs* 59 years, P = 0.022) and more likely to be male (57 *vs* 55%, P = 0.448) compared to the non-HCC patients (n = 456). Furthermore, higher hepatic enzymes levels, ActiTest scores, and lower PLT counts were observed at baseline in the HCC patients than the non-HCC patients.

Table 2.XXVII presents the variables detected by multivariate Cox hazard regression analysis, which are significantly associated with the HCC development. According to the AUROC analysis for the HCC development regarding the AFP level recorded in all patients, the best cutoff value for the AFP level assessed at DAAs cessation was 10 ng/mL. After adjusting the intervening variables, we found an HR of the HCC development with a serum AFP level at EOT > 10 ng/mL of 3.01 (95% CI, 1.092 - 8.340, P = 0.046). A serum AFP level at EOT> 10 ng/mL was an independent risk factor of the HCC occurrence.

HCC Features and Management. Of the patients who developed HCC, 12 patients (52.2%) met the Milan criteria, and according to the BCLC classification, a majority of them were in class A (39.1%) (Table 2.XXVIII). Regarding the imaging characteristics of the tumor, most patients had a single nodule (65.2%), and the presence of the capsule was highlighted in most cases (82.6%). Malignant PVT was demonstrated in 26% of the patients, and local/distant metastases was noticed in 13% of the patients.

Table 2.XXVII. Cox regression analysis for HCC occurrence

Variables	**Multivariate Analysis**		
	HR	**95% CI**	**p-Value**
Male (gender)	1.25	0.866 – 1.825	0.278
Age > 60 years	2.08	1.409 – 3.066	0.002
Obesity	1.32	0.182 – 9.577	0.792
Treatment experienced	1.53	0.981 – 2.404	0.094
AFP EOT > 10 ng/mL	3.01	1.092 – 8.340	0.046
Abbreviations: AFP, alpha-fetoprotein; EOT, end of treatment			

Table 2.XXVIII. HCC classification and imaging features

Variables	**HCC cases (n = 23)**
Milan	
Within	12 (52.2)
Beyond	11 (47.8)
BCLC	
A	9 (39.1)
B	4 (17.4)
C	7 (30.4)
D	3 (13)
Imaging Feature	
Single tumor	15 (65.2)
Capsule visualization	19 (82.6)
Metastasis	3 (13)
Malignant PVT	6 (26)
Abbreviations: BCLC, Barcelona clinic liver cancer; PVT, portal vein thrombosis. [a] Values are expressed as No. (%)	

The therapeutic management of the patients with HCC was performed according to the BCLC classification. Most patients (n = 11, 47.8%) received curative treatment by surgical resection, among whom histopathological examination detected a moderately differentiated in five patients, a poorly differentiated tumor in five patients, and a well-differentiated tumor only in one patient. Three patients (27%) had HCC recurrence after a mean of 65.2 months sand were subsequently addressed for systemic therapy.

Discussion. The high rate of SVR, along with an excellent tolerability profile and extended indication for antiviral treatment at all stages of chronic HCV infection, enhanced expectations for lower HCC rates after viral eradication with the DAA therapy among clinicians worldwide. Such enthusiasm was overshadowed after the publication of data indicating, in contrast to expectations, unexpectedly higher novo and recurrent HCC after DAA treatment in patients with chronic HCV infection (Reig et al., 2016; Conti et al., 2016). These findings have led to the emergence of an "avalanche" of studies assessing this risk.

Contrary to our expectations, we observed the sustained HCC incidence rates for up to 5.1 follow-up years in HCV-free cirrhotic patients after the DAAs treatment. The cumulative 5-year risk was 2.6%, and this value exceeded the cutoff beyond which HCC surveillance was cost-effective (Kanwal et al., 2020).

Nonetheless, similar to the findings of other studies, the findings of the present study support the incidence of HCC, with no evidence on the high occurrence of de novo HCC after the DAA therapy. For example, a prospective study by Cheung et al. (Cheung et al., 2016) revealed no increased risk of HCC during or 12 months after the DAA cessation in 406 patients with HCV-related decompensated cirrhosis. Furthermore, the researchers concluded that the HCC incidence of 4.2% observed during the first six months after DAAs was identical to the incidence rate in the untreated control group. Similarly, a cohort study on 22,500 DAAs treated patients reported a significant decrease in the HCC risk in patients who achieved SVR compared to the non-responders (0.90 vs. 3.45 cases of HCC/100 person-years) (Kanwal et al., 2017). Interestingly, the same authors in their later article on DAAs-treated patients, who were followed up over 3.5 years after SVR, reported the 1-, 2-, and 3-year cumulative risks of HCC to be 1.1, 1.9, and 2.8%, respectively (Kanwal et al., 2020).

In contrast to the interferon era, the increased efficacy and tolerability of DAA allowed treatment in patients with more risk factors for HCC than patients in historical cohorts treated with interferon, the most relevant of which were older age, diabetes mellitus, and liver cirrhosis. Regarding the presence of risk factors, the existence of inhomogeneity in the DAAs and interferon groups may explain the higher incidence rates of HCC in patients treated with the new antivirals.

Recent data have indicated that the risk factors associated with de novo and recurrent HCC after DAA treatment are represented by older age, advanced liver fibrosis, and the absence of SVR.

In the present study, age > 65 years at baseline and a cutoff value of AFP at EOT = 10 ng/mL were independent risk factors associated with the HCC occurrence. Although there are abundant data regarding the HCC occurrence and recurrence after DAAs, few reports assessed the behavior of HCC and access to curative therapy after the DAAs treatment (Nakao et al., 2018; El Fayoumie et al., 2020; Fatima et a.l, 2020). Regarding tumor aggression, the patients with HCC in the present study revealed aggression less frequently than those reported in other studies. For example, a recent study by Fayoume et al. demonstrated that the frequency of cases with multiple HCC and infiltrative HCC was significantly higher among the DAA-treated patients than in naïve patients with HCC (El Fayoumie et al., 2020). In contrast, most HCC patients in the present study had a single nodule (65.2%). The portal vein invasion was observed in 26% of patients, and there were local and distant metastases in 13% of the patients. Furthermore, according to the BCLC classification, most patients were in classes O and A; thus, they had access to curative treatment. Similarly, Fatima et al. reported that, considering the BCLC stages, multiplicity, malignant PVT, and local spread through malignant lymphadenopathy, the HCC pattern did not differ between patients treated with IFN and those treated with DAAs (Fatima et al., 2020).

The main limitation of the present study was the presence of no control group of untreated patients to compare the characteristics and the prognosis of HCC after DAAs. Other

limitations were the lack of patients with decompensated cirrhosis and the use of a single DAAs regimen in our cohort.

Conclusions. The present study revealed no evidence of the high HCC occurrence after long-term follow-up of patients with HCV genotype 1b infection and liver cirrhosis, who achieved SVR following the DAAs treatment. However, the cumulative 5- year risk remained above the cutoff point, above which the HCC screening becomes cost-effective. According to these findings, clinicians should maintain HCC surveillance in those with liver cirrhosis at the time of SVR. The tumor phenotype does not seem to be more aggressive after DAAs, and the access to curative therapy is similar to that of the HCC associated with other liver diseases. The evaluation of the HCC pattern requires prospective case-control studies comparing the clinical-biological and imaging markers of tumor aggression between the HCC cases after DAAs and naïve patients.

2.7.3. No evidence of a more aggressive pattern of hepatocellular carcinoma after Direct Acting Antivirals-results from a single center observational study

Aim - to identify and characterize the changes in the pattern and imaging features of HCC developed after DAAs therapy in patients with chronic HCV infection and liver cirrhosis, as well as the impact on HCC treatment decisions, compared to patients with HCC and HCV- related liver cirrhosis without previous antiviral treatment.

Materials and methods. This is a single-center case-controlled retrospective study in which consecutive patients with HCC and HCV-related liver cirrhosis admitted in a tertiary gastroenterology referral center from North-Eastern Romania, between January 1st, 2016 and May 1st, 2018, were included. All patients who presented during the same period were divided into 2 groups depending on the DAAs-treated (group I) or untreated status (group II). All patients with other concomitant causes of liver disease such as autoimmune hepatitis, thesaurismosis, non- alcoholic fatty liver disease, HCV/HBV and HCV/HIV co-infection were excluded. The regimens used in treated patients were: PrOD in patients with HCV-related compensated liver cirrhosis and Ledipasvir/Sofosbuvir (LED/SOF) +RBV for 12 weeks, in patients with decompensated liver cirrhosis.

The HCC tumor pattern was evaluated in all patients and consisted of the quantification of the largest tumor dimension, HCC multiplicity, the presence of malignant PVT, and AFP levels.

Statistical analysis. Data collected was statistically analyzed using SPSS 20.0 (Chicago, IL, SUA). All tests were two-tailed with p-value <0.05 considered statistically significant. Continuous variables were expressed as mean ±standard deviation for normally distributed continuous data, and as median for non- normally distributed continuous data. Groups were compared using Chi square test or Fisher's exact test for categorical variables and using independent t-Student test or Mann-Whitney U test for continuous variables (depending on data distribution).

Results. This study included 69 patients diagnosed with HCC and HCV-related liver cirrhosis, mean age 67.5±12.2 years, mostly males 53.6%. Thirty-eight (55.1%) patients had comorbidities such as arterial hypertension (42%), diabetes (18.8%) and obesity (11.6%). Most patients had decompensated liver cirrhosis (55.1%), with a mean Child Pugh score of 7.4±0.25.

The patients were divided into 2 groups, depending on the DAAs-treated or untreated status, as follows: group I included 15 patients with HCC and HCV-related liver cirrhosis who were previously treated with DAAs, whereas group II consisted of 54 patients who were admitted in the same period with HCC and HCV-related liver cirrhosis without evidence of previous antiviral treatment.

The majority of patients from group I were females (53.4%), with mean age of 65.1±9.8 years. The majority had compensated liver cirrhosis; 86.6% had Child-Pugh A class, whereas 13.4% only were decompensated (1 patient had Child-Pugh B and 1 patient Child-Pugh C) (Table 2.XXIX).

Eleven (73.4%) patients were previously treated with PrOD and 4 (26.6%) patients received LED/SOF+RBV. SVR rate in the studied patients in group I was 100% and was defined by undetectable HCV-RNA at 12 weeks after end of treatment (EOT). Patients in group II were predominantly males (55.5%), with similar mean age as group I (68.1±12.8), but with a higher percentage of decompensated liver cirrhosis than group I (Child-Pugh score was 5.9±1.4 in group I vs. 7.8±2.1 in group II, p=0.002). With respect to liver function, hepatocytolysis was more significant in group II compared to group I (ALT: 112.2 IU in group II vs. 41.6 IU in group I, p= 0.049; AST 70.4 IU in group II vs. 37.1 IU in group I, p=0.028).

Table 2.XXIX. Demographics and clinical characteristics in the study group

Parameter	DAAs treated N=15 (Group I)	No DAAs N=54 (Group II)	p-Value
Age (yrs.), mean ± SD	65.1±9.8	68.1±12.8	0.397
Male, n (%)	7 (46.6)	30 (55.5)	0.541
Child-Pugh class, n (%)			**0.001**
A	13 (86.6)	18 (33.3)	
B	1 (6.6)	22 (40.7)	
C	1 (6.6)	14 (25.9)	
Child-Pugh score, mean ± SD	5.9±1.4	7.8±2.1	**0.002**
FIB4, mean ± SD	3.4±2.3	33.5±25.1	0.536
APRI, mean ± SD	0.84±0.5	2.4±2.1	**0.004**
Comorbidities, n (%) Arterial hypertension Diabetes mellitus Obesity	8 (53.3) 7 (46.6) 3 (20) 3 (20)	30 (55.5) 22 (40.7) 11 (20.3) 5 (9.2)	0.878 0.681 0.538 0.250
ALT (UI/L), mean ± SD	41.6±19.2	112.2±13.2	**0.049**
AST (UI/L), mean ± SD	37.1±25.1	70.4±13.5	**0.028**

Abbreviations: IQR, interquartile range; SD, standard deviation;
FIB4, Fibrosis-4 Index for Liver Fibrosis; APRI, AST to Platelet Ratio Index.

Regarding the distribution of hepatic focal lesions, there were no significant differences between the two groups (73.3% of group I patients and 55.5% of group II had unicentric lesion, p=0.215) (Table 2.XXX).

Table 2.XXX. Tumor characteristics in the studied patients

Parameter	DAAs treated N=15 (Group I)	No DAAs N=54 (Group II)	p-Value
AFP, mean ± SD	163.1±42.1	602.8±84.1	0.059
BCLC stage, n (%)			
A	5 (33.3)	15 (27.7)	
B	6 (40)	13 (24.1)	0.481
C	2 (13.3)	14 (25.9)	
D	2 (13.3)	12 (22.2)	
Within Milan criteria, n (%)	10 (66.6)	19 (35.2)	**0.029**
Unicentric, n (%)	11 (73.3)	30 (55.5)	0.215
Tumor size < 3cm, n (%)	10 (66.6)	16 (29.6)	**0.009**
PVT, n (%)	2 (13.3)	24 (44.4)	**0.028**
Lymph node metastasis, n (%)	1 (6.66)	13 (24%)	**0.031**

Abbreviations: IQR, interquartile range; SD, standard deviation; PVT, portal vein thrombosis.

The tumor size was significantly higher in group II compared to group I, with 66.6% of patients in group I and only 29.6% of patients in group II that have a tumor size less than 3 cm (p=0.009). AFP level was higher but not statistically significant in group II with a median (IQR) value of 78 ng/mL compared with a median (IQR) value of 8.4 ng/mL (32.7) in group I (p=0.059).

An aggressive tumor pattern was established based on the identification of malignant adenopathy in 1 patient and malignant PVT in 2 patients from group I, and in 13 and 24 patients, respectively in group II, with a statistically significant difference between the two groups (p=0.031 and p=0.028, respectively). None of the studied patients developed another distant metastasis. According to BCLC classification, the majority of patients in group I were in stage A (33.3%) and B (40%), whereas patients in group II were approximately equally distributed in all stages (A-27.7%, B-24.1%, C-25.9%, and D-22.2%). With respect to Milan criteria, 6.6% of patients from group I and only 35.2% of patients from group II were within the criteria (p=0.029). Aggressive pattern of HCC had its impact on therapeutic decision. Supportive treatment without ablation was more common in group II (33.3%) than group I (6.6%) (p=0.047). Otherwise, transarterial chemoembolization was the most frequent choice followed by surgery in both groups. Only one patient (1.8%) from group II had liver transplant.

Discussion. Although there is a high amount of data regarding HCC occurrence and recurrence after DAAs, few reports assessed the behavior of HCC and the access to curative therapy after DAAs treatment. Data from our study emphasis important findings regarding the behavior of HCC after DAAs therapy compared to naïve patients. The incidence rates of HCC in patients without previous antiviral therapy seems to be related to the severity of cirrhosis. Furthermore, patients from group I had a higher percentage of compensated cirrhosis than those from group II (86.6% vs. 33.3%, p=0.001).

Recent studies which evaluated the behavior of HCC after DAAs, have found more aggressive features of HCC. For instance, a recent article by Fayoume et al. found that multiplicity and infiltrative pat- terns were more common in DAAs-treated HCC patients compared to naïve HCC patients (El Fayoumie et al., 2020). Another study revealed that malignant PVT and lymphadenopathy were significantly more frequent in DAAs- treated HCC patients (Khalid J, et al, 2020). On the contrary, we found a more aggressive behavior of HCC among untreated patients from group II compared to group I. Although the lesions were predominantly unicentric in both groups, we found that malignant PVT and lymphadenopathy were more frequent in group II than in group I.

Furthermore, patients from group I met the Milan criteria more frequently and had smaller sizes of tumor lesions than those from group II. According to BCLC classification, we found no significant differences between the two groups. Similarly, Fatima et al. reported that the pattern of HCC did not differ between patients treated with IFN- based therapy and those DAAs-treated, considering BCLC stages, multiplicity, malignant PVT and lymphadenopathy (Fatima et al., 2020). The best supportive care was the only recommended line of treatment for 6.6% patients from group I and for 33.3% patients from group II. The significantly higher percentage of patients from group II that received only best supportive care reflects the limited access to therapy based on BCLC classification. Access to curative therapy by surgical ablation was better in group I vs. group II, although TACE and sorafenib were the next most frequent options in both groups.

Conclusions. Our study found no evidence of a more aggressive behavior of HCC after DAAs treatment compared to naïve patients. On the contrary, we found a better access to HCC curative therapy in this group compared to untreated patients.

Based on these findings, the screening of HCC in patients with HCV-related liver cirrhosis remains necessary after achieving viral clearance with DAAs therapy. According to current guidelines, abdominal ultrasound and AFP monitoring every 6 months in HCV-related liver cirrhosis seems the most appropriate strategy for these patients.

Advanced liver disease

LC is a major health problem worldwide and most of its complications are often life threatening. Despite the scientific and therapeutic progress we are sure that this pathology will continue be a therapeutic challenge for a long time.

In the absence of transplanted liver the evolution of most cirrhotic patients is unfortunate but I am convinced that in the near future we will be able to do much more for these patients than just keep them alive.

Synthesis of scientific contributions
- With the increase in life expectancy, we have more and more elderly patients with LC. Primary and secondary prevention of VB can be a challenge in these patients because many of them have contraindications for beta-blockers.
- The increased systemic oxidative stress in HE cirrhotics patients is suggested by decrease of both, antioxidants enzymes activity, SOD and GPx, as well as by the significant increase of MDA serum levels - Oral glutamine load improves the performance of psychometric tests for the diagnostic of MHE
- SBP secondary prophylaxis should be personalized in order to outweigh the risks associated with the CDI development.
- VA presents a good predictive value for in-hospital mortality in patients with ACLF and high levels of VA are associated with severe HE. - VA has the potential to be used as an additional prognostic marker in the evaluation of patients with ACLF.
- HCC phenotype does not seem to be more aggressive after DAAs, and the access to curative therapy is similar to that of the HCC associated with other liver diseases - The screening of HCC in patients with HCV-related liver cirrhosis remains necessary after achieving viral clearance with DAAs therapy.

Chapter 3

CLOSTRIDIUM DIFFICILE INFECTION IN GASTROENTEROLOGY AND HEPATOLOGY – *DIFFICILE* INFECTION?

3.1. INTRODUCTION

Although most often the clinical forms of *Clostridium difficile* colitis are mild/moderate and respond to the initial treatment there are also patients with CDI with severe clinical presentations, frequently requiring intensive care unit admission (Marra et al., 2020). Unfortunately, not infrequently CDI occurs in patients hospitalized in gastroenterology and hepatology departments with advanced liver disease, inflammatory bowel disease, digestive neoplasia, immunocompromised patients, often elderly, etc. All of these patients may become critical at some point either due to the CDI itself or the aggravation of the underlying disease precipitated by the CDI. Approaching a patient with severe CDI or a patient with multiple comorbidities remains a challenge for clinicians.

C.difficile, also known as *Clostridioides difficile* is an anaerobic, gram-positive, spore forming bacterium transmitted via the fecal-oral route. *It* was first isolated from the stool of a healthy infant by Hall and O'Toole in 1935 (Hall and O'Toole, 1935) and the name was chosen to reflect the *difficulty* with its culture and isolation. Certainly, the godparents of this microorganism did not imagine that the name will remain relevant over time current both in terms of diagnosis and treatment of infection. The diagnosis of CDI is often very simple but can be difficult (*difficile*) in patients with atypical symptoms; the treatment of the first infection is often efficient but can be extremely difficult (*difficile*) and challenging in patients with relapses of the infection.

Since 1978, when *C. difficile* was found to be the cause of pseudomembranous colitis (Song and Kim, 2019; Doll et al., 2021), n*umerous epidemiological data showed that* CDI is the leading cause of nosocomial infectious diarrhea worldwide (Ponce-Alonso et al., 2019; Marra et al., 2020) and one of the most common health care-associated infections (Magill et al., 2014).

Over the two last decades there has been a dramatic worldwide increase in both incidence and severity of CDI (Wiuff et al., 2018). In the United States, CDI caused about half a million infections and almost 30.000 annual deaths (Lessa et al., 2015); in Europe about 152.905 persons were infected with *C. difficile* with an annual mortality above 8,000 persons (De Roo et al., 2020, Malfertheiner et al., 2020).

The prevention for CDI difficile remains a significant concern for the health system trying to prevent outbreaks and maximize patient safety. As one of the first definitions of CDI was associated to antibiotic colitis the first and most widely recognized causative factor for CDI is the broad-spectrum antimicrobial therapy (Liang et al., 2020; Granata et al., 2020; Chen et al., 2020).

Although the majority of literature on the epidemiologic features of CDI is based on association with antibiotic therapy and hospitalization settings (Laszkowska et al., 2021) some other potential risk factors for CDI, such as advanced age, immunosuppression, comorbidities, chemotherapy, renal insufficiency, hypoalbuminemia, organ transplantation, use of PPI have been identified to explain the increased incidence of CDI (Lessa et al., 2015; Ferreira et al., 2020; Spigaglia et al., 2020). Moreover, it should be noted that rates of community-associated *C. difficile* infections appear to be increasing worldwide (Aguila et al., 2020).

In the early 2000s, there was an alarming rate of increase of *C. difficile* infection with increasingly severe disease due to epidemic NAP1 1/027 strain (North-American Pulsed-Field Type 1), a new hypervirulent and easily transmissible strain in some North American and European areas (Wiuff et al., 2018; Doll et al., 2021).

The clinical spectrum of CDI ranges from an asymptomatic carriage to fulminant colitis with toxic megacolon. Watery diarrhea is the cardinal symptom in CDI and, therefore, if this symptom is present, especially in relation with the use of antibiotics, CDI must be suspected and any physician should ask a stool test for *C. diffcile*.

The increased incidence of CDI, the risk of recurrence, difficult treatment in relapses are associated with high economic costs, which burden the health system worldwide (Marra et al., 2020). The prevention for CDI remains a significant concern for the health system trying to prevent outbreaks and maximize patient safety.

In 2011 we have published the first case of CDI in Romania, in a 72-years old man with diarrhea, hypoproteinemia, edema and ascites (Cojocariu et al., 2011).

Since then, this topic has been a concern in my scientific and research work in the following areas:

- awareness about the importance of CDI,
- implications of CDI in patients with inflammatory bowel disease,
- CDI in patients with advanced liver disease,
- CDI in geriatric patients,
- last but not least, in the current period of the severe acute respiratory syndrome coronavirus 2 (SARS CoV 2) pandemic, we were concerned with analyzing the incidence of CDI in patients with Covid infection.

Personal contribution related to CDI in gastroenterology and hepatology is synthesized in the following papers:

	Articles published in ISI indexed journals	**WOS citations**
1.	**Cojocariu C**, Danciu M, Chiriac S, Trifan A, Stanciu C. A 72-year old man with diarrhea, hypoproteinemia, edema and ascites. *J Gastrointestin Liver Dis* 2011; 20: 314. **IF – 2.202**	**3**
2.	Anca Trifan, Irina Girleanu, **Camelia Cojocariu**, Catalin Sfarti, Ana Maria Singeap, Carmen Dorobat, Lucia Grigore, Carol Stanciu. Pseudomembranous colitis associated with a triple therapy for *Helicobacter pylori* eradication. *World J Gastroenterol*. 2013 November 14; 19(42): 7476-7479. doi: 10.3748/wjg.v19.i42.7476. **IF 2.433**	24
3.	Trifan A, Stoica O, Stanciu C, **Cojocariu C**, Singeap AM, Girleanu I, Miftode E. *Clostridium difficile* infection in patients with liver disease: a review. *Eur J Clin Microbiol Infect Dis.* 2015 Dec;34(12):2313-24. doi: 10.1007/s10096-015-2501-z. **IF 2.72**	24
4.	**Cojocariu C**, Girleanu I, Trifan A, Olteanu A, Muzica CM, Huiban L, Chiriac S, Singeap AM, Cuciureanu T, Sfarti C, Stanciu C. Did the severe acute respiratory syndrome-coronavirus 2 pandemic cause an endemic *Clostridium difficile* infection? *World J Clin Cases*. 2021;26;9(33):10180-10188. **IF – 1.33**	
5.	Irina Girleanu**,** Anca Trifan, Laura Huiban, Cristina Muzica, Roxana Nemteanu, Andreea Teodorescu, Ana Maria Singeap, **Camelia Cojocariu**, Stefan Chiriac, Oana Petrea, Sebastian Zenovia, Robert Nastasa, Tudor Cuciureanu, Carol Stanciu. The risk of *Clostridioides difficile* infection in cirrhotic patients receiving Norfloxacin for secondary profilaxis of spontaneous bacterial peritonitis- A real life cohort. *Medicina* (Kaunas) 2021; 57(9):964 **IF- 2.43**	
6.	Trifan A, Girleanu I, Stanciu C, Miftode E, **Cojocariu C**, Singeap AM, Sfarti C, Chiriac S, Cuciureanu T, Stoica O. *Clostridium difficile* infection in hospitalized octogenarian patients. *Geriatr Gerontol Int*. 2018 Feb;18(2):315-320. https://doi: 10.1111/ggi 13186 PMID: 29139189. ISSN: 1447-0594. **IF- 2.35**	7
7.	Anca Trifan, **Camelia Cojocariu**, Oana Stoica, Carol Stanciu. The epidemiology of *Clostridium difficile* infection in Romania: what we know, or do not know and why? *J Gastrointestin Liver Dis*, March 2014 Vol. 23 No 1: 99-104 **IF 1.45**	4

Articles published in ISI indexed journals		WOS citations
8.	**Cojocariu C**, Stanciu C, Stoica O, Singeap AM, Sfarti C, Girleanu I, Trifan A. *Clostridium difficile* infection and inflammatory bowel disease. *Turk J Gastroenterol*. 2014 Dec;25(6):603-10. doi: 10.5152/tjg.2014.14054. Review. **IF 0.75** doi: 10.5152/tjg.2014.14054.	**11**
9.	Anca Trifan, Carol Stanciu, Oana Stoica, Irina Girleanu, **Camelia Cojocariu**. Impact of *Clostridium difficile* infection on inflammatory bowel disease outcome: A review. *World J Gastroenterol*. 2014 September 7; 20(33): 11736-11742. doi: 10.3748/wjg.v20.i33.11736. **I**F **2.43**	36
10.	Stoica OC, Stanciu C, **Cojocariu C**, Miftode E, Boiculese L, Trifan A, Girleanu I. *Clostridium difficile* Infection in Hospitalized Cirrhotic Patients with Hepatic Encephalopathy. *J Gastrointestin Liver Dis*. 2015 Dec;24(4):423-8. doi: 10.15403/jgld.2014.1121.244.csd. PMID:26697567. **IF 1.837**	9
11.	**Cojocariu C**, Stanciu C, Ancuta C, Danciu M, Chiriac S, Trifan A. Immunoglobulin A Vasculitis Complicated with *Clostridium difficile* Infection: a Rare Case Report and Brief Review of the Literature. *J Gastrointestin Liver Dis*. 2016 Jun;25(2):235-8. doi: 10.15403/jgld.2014.1121.252.csd. PMID:27308656. **IF – 1.71**	**9**
Articles published in extenso in BDI indexed journals		
12.	**Camelia Cojocariu,** Anca Trifan, Oana Stoica, C.A. Chihaia, C. Stanciu. *Clostridium Difficile* Infection and Inflammatory Bowel Disease: What Gastroenterologists and Surgeons Should Know. *Chirurgia* 2014 (109):No. 5, September – October; 579-583	
13.	Stoica O, Trifan A, **Cojocariu C**, Gîrleanu I, Maxim R, Stanciu C. Incidence and risk factors of *Clostridium difficile* infection in patients with inflammatory bowel disease. *Rev Med Chir Soc Med Nat Iasi*. 2015 Jan-Mar;119(1):81-6. PMID: 25970947	9
Articles published in published in extenso in ISI proceedings journals		
1.	Oana Cristina Petrea, **Irina Girleanu,** Ana Maria Singeap, Camelia Cojocariu, Laura Huiban, Cristina Muzica, Stefan Chiriac, Anca Trifan. *Clostridium difficile*: False or true alarm? *Proceedings of the Central European Gastroenterology Meeting (CEURGEM)* 2019; 97-102	
Book chapters		
1.	Oana Stoica, Anca Trifan, **Camelia Cojocariu**, Irina Gîrleanu, Carol Stanciu. Particularităţile infecţiei cu *Clostridium difficile* în bolile inflamatorii intestinale. In C Stanciu, A Trifan, I Sporea. *Bolile inflamatorii intestinale*, Ed "Gr T Popa", Iasi, 2014: 265-275. ISBN 978-606-544-220-7.	
Total WOS citations		**136**
Cumulative impact factor / direction		**21.642**

3.2 AWARENESS ABOUT *CLOSTRIDIUM DIFFICILE* INFECTION

I have listed the published papers related to CDI, but I think that our most important collective contribution regarding CDI is the concern expressed in our first article (Cojocariu et al., 2011) and the awareness (proven to be successful) about this pathology, which in last years is a real health problem burdening health system worldwide.

In 2011 (the same year of our case report) one hospital-based survey regarding CDI in Europe (in which 5 Romanian hospitals participated together with 97 hospitals from 34 European countries) showed a very low incidence of CDI in Romania (Bauer et al., 2011). We have some doubts that the results are representative for the incidence of CDI in Romania and we pointed out that the main reason for these data is the lack of awareness about the pathology in our country; thus, only 3 Romanian patients per 10,000 patient-days were tested as compared to 38 from Hungary, 115 from the UK, or 141 from Finland (Bauer et al., 2011).

Three years later the EUCLID study reported an underdiagnosis of CDI more than 60% in Romania (compared to 0% in Belgium, the Netherlands and Sweden). The main reasons for the high CDI underdiagnosis are related to the technical endowment (laboratory diagnostics,

previous lack of consensus on optimal testing methodology, etc.) but especially to the ignorance and lack awareness of CDI (Davies et al., 2014).

I would like to emphasize our contribution to raising awareness about CDI because most cases were underdiagnosed, either due to low levels of awareness for CDI among clinicians or misdiagnosed in the absence of sensitive diagnostic tests. Illustratively, in our institution, after the first case diagnosed with pseudomembranous colitis in 2011 (Trifan et al., 2014), number of tests ordered for *C. diffcile* by our physicians and, subsequently, patients diagnosed with CDI have significantly increased (Figure 3.1) (Stoica, 2017).

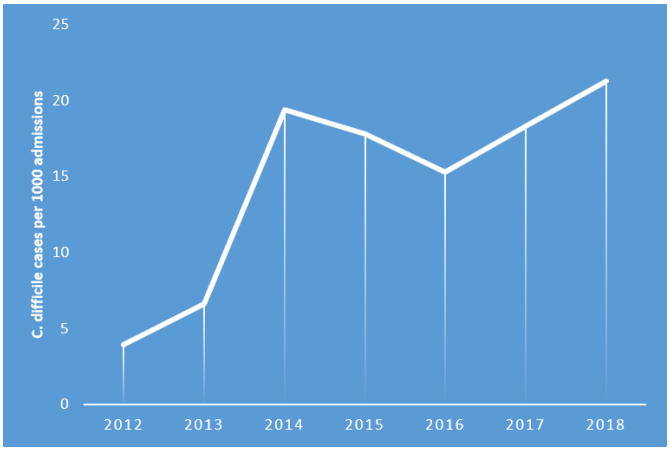


Fig. 3.1. Increasing rates of *C. difficile* infection among patients hospitalized at Institute of Gastroenterology and Hepatology, Iasi, Romania

Since 2011 we pointed out that the fight to *C. difficile* is difficult, but we can achieve success only by increasing clinical suspicion for CDI in patients with unexplained watery diarrhea, systematically implementing guidelines in our routine clinical practice, as well as supporting education at a wide level (Trifan et al., 2014).

Overlapping CDI can sometimes make it difficult to diagnose an autoimmune disease and we have presented a very rare case of IgA vasculitis complicated by CDI. To the best of our knowledge, this was the second published case of IgA vasculitis complicated by CDI in adults (Cojocariu et al., 2016) (Figure 3.2).

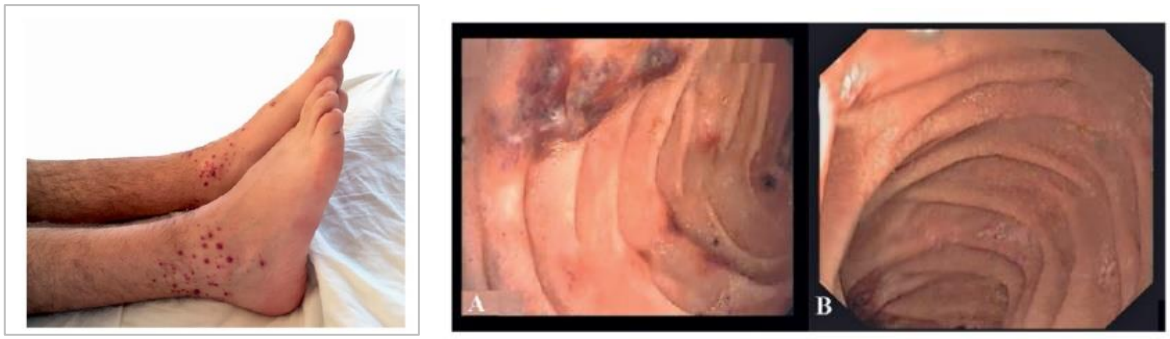


Fig. 3.2. Palpable purpura on both ankles and endoscopic appearance of the second part of the duodenum: A) (before treatment): multiple erosions, diffuse redness, submucosal hemorrhage, petechiae, small ulcerations; B) (after treatment): significant improvement of endoscopic appearance

The CDI in our patient may have been caused by the changes in gut microbiota induced by antibiotic therapy administered prior to hospital admission and the corticosteroids administered for the treatment of the IgA vasculitis. The association of CDI can mask the diagnosis of an autoimmune disease but at the same time can be a challenge for the treatment of patients with autoimmune diseases.

The implications of CDI in patients with autoimmune diseases is a challenging topic, which we intend to study in the future.

We wanted to raise awareness about CDI primarily gastroenterologists but also surgeons, especially in inflammatory bowel disease (IBD) patients. CDI has a negative impact both on short- and long- term IBD outcomes, increasing the need for surgery, as well as the mortality rate and healthcare costs. All gastroenterologists and surgeons should have a high index of suspicion for CDI when evaluating a patient with IBD flare, as prompt diagnosis and adequate treatment of infection improve outcomes. Measures must be taken to prevent spreading of infection in gastroenterology /surgery settings (Cojocariu et al., 2014).

3.3. *CLOSTRIDIUM DIFFICILE* INFECTION AND INFLAMMATORY BOWEL DISEASE

3.3.1. Scientific contents

Paralleling to increasing incidence of CDI in the general population, there has been an even higher increase in incidence of CDI among patients with inflammatory bowel disease (Issa et al., 2007; Nguyen et al., 2008; Bossuyt et al., 2009; Ricciardi, 2009;. Jen et al., 2011). CDI was first associated with IBD in the early 1980s (LaMont, et al., 1980), but was somehow neglected until fifteen years ago, when several studies and reviews clearly demonstrated that patients with one of the two major forms of IBD (ulcerative colitis-UC; Crohn's disease-CD) are at high-risk for CDI (Navaneethan et al., 2010; Ananthakrishnan et al., 2010; Saidel-Odes et al., 2011; Goodhand et al., 2011; Sinh et al., 2011; Reddy et al., 2013; Nitzan et al., 2013; Berg et al., 2013).

Besides the well-known risk factors for CDI in non-IBD population, there are some typical IBD-related risk factors, such as the extent and severity of the underlying disease, long-term immunosuppression, lack of antibiotic exposure or recent hospitalization, younger age, steroid use, and predominantly community-acquired infection (Issa et al., 2007; Rodemann et al., 2007). Patients with UC are more susceptible to CDI than those with CD (Issa et al., 2007; Rodemann et al., 2007; Nguyen et al., 2008; Ricciardi et al., 2009; Nitzan et al., 2013). CDI may resemble a flare of IBD as symptoms are often similar, and consequently screening for CDI is recommended at every flare in such patients (Navaneethan et al., 2010; Ananthakrishnan et al., 2010; Saidel-Odes et al., 2011; Sinh et al., 2011; Reddy et al., 2013; Nitzan et al., 2013; Berg et al., 2013).

For a long time been found that patients with *C. difficile* and IBD had four times greater mortality than patients admitted for IBD alone and two times greater mortality than patients admitted for *C. difficile* alone (Ananthakrishnan et al., 2008).

Risk factors. In addition to the traditional risk factors for CDI similar to those in the general population, there are some IBD-specific risk factors for CDI.

Traditional risk factors, of which prior antibiotics use remains the most important, include advanced age, multiple comorbidities, prolonged hospitalization, and immunosuppression. Clindamycin was initially associated with CDI, but more recently, fluoroquinolones, widely used in IBD patients, have become the most common risk factor for CDI (Marwick et al., 2013). The mechanism through which the use of antibiotics leads to increased risk for CDI development is the disruption of normal intestinal flora and the subsequent proliferation of *C. difficile*. However, in IBD patients, antibiotics do not play such an important role in CDI development as they do in the general population (Issa et al., 2007; Bossuyt et al., 2009). One study (Bossuyt et al., 2009) reported that antibiotic use before the development of CDI was found in only 40% of IBD patients as compared to 69% in non-IBD

patients, while another study identified no recent antibiotic use in 39% of IBD patients with CDI (Issa et al., 2007).

As in the general population, advanced age and comorbidities increase the risk for CDI in IBD patients (Rodemann et al., 2007; Ananthakrishnan et al., 2011). Although the proportion of older IBD patients has increased in recent years, a nationwide retrospective study found an average age of CDI in IBD cohorts significantly lower than in general population controls (Jen et al., 2011). Nguyen et al. (Nguyen et al., 2008) reported a 13% increase in the risk for CDI with each 1-point increase in the Charlson's comorbidity burden index. Prolonged and frequent hospitalizations are a risk factor for CDI in both IBD and non-IBD patients, although several studies have reported that CDI in IBD patients is often acquired outside the hospital (Rodemann et al., 2007; Issa et al., 2007).

IBD patients often require long-term immunosuppressive therapy, which is another risk factor for CDI both in IBD and non-IBD patients (e.g., organ transplant recipients). Issa et al. (Issa et al., 2007) found maintenance immunosuppressive therapy to be associated with a double risk of CDI (OR 2.58; CI:1.28-5.12). However, other studies found no such association (Kariv et al., 2011). Studies on immunosuppression with corticosteroids have also reported conflicting results, some finding no association with the risk of CDI in IBD patients, others reporting an increased risk of CDI (Schneeweiss et al., 2009). Schneeweiss et al. (Schneeweiss et al., 2009) found that corticosteroid initiation tripled the risk of CDI among the IBD patients (relative risk 3.4, 95% CI 1.9-6.1), while no such association with the initiation of immunomodulators or biologics (infliximab) was found.

Biological agents (e.g., infliximab, adalimumab) are not associated with increased risk of CDI in IBD patients (Schneeweiss et al., 2009), nor is the use of aminosalicylates (Clayton et al., 2009). PPI use in non-IBD patients has been suggested to increase the risk for CDI (Trifan et al, 2013), but other studies did not find any association between PPI use and CDI in IBD patients (Bossuyt et al., 2009).

IBD-specific risk factors. Firstly, IBD it self is an independent risk factor for CDI, with a three-fold increased risk as compared with non-IBD population (Rodemann et al., 2007). Patients with UC are at higher risk for CDI than those with CD (Rodemann et al., 2007; Issa et al., 2007; Nguyen et al., 2008), and colonic CD patients are at higher risk for CDI than those with small bowel CD (Issa et al., 2007; Nguyen et al., 2008). IBD patients with extensive disease and greater disease activity are at higher risk for CDI (Issa et al., 2007).

Challenges of CDI in IBD patients

Watery diarrhea is the cardinal clinical symptom of CDI. However, CDI in IBD patients may show atypical features including bloody or mucous diarrhea, which are difficult to distinguish from an IBD flare (Issa et al., 2007; Nguyen et al., 2008). In many patients there are associated systemic symptoms, such as low-grade fever or anorexia. Laboratory findings in CDI and IBD flares are also similar (leukocytosis, anemia, hypoalbuminemia). Moreover, at colonoscopy pseudo membranes are often absent in IBD patients with CDI (Ben-Horin et al., 2009).

Which IBD-patients should be tested for *C. difficile?* Due to the high prevalence of CDI in IBD patients and clinical/laboratory similarity between CDI and IBD exacerbation, it is recommended that all IBD patients with a disease flare should be tested for *C. difficile* (Navaneethan et al., 2010; Sinh et al., 2011; Nitzan et al., 2013; Berg et al., 2013). There is no indication for post-treatment testing to confirm cure. Asymptomatic carriage in IBD patients (remission/inactive disease) is more frequent than in general population. Screening for asymptomatic carriers in the general population is not recommended (Clayton et al., 2009),

but screening and treatment of asymptomatic IBD patients under immunosuppressant therapy may be important (Issa et al., 2007).

IBD patients with CDI are under a higher risk of worse outcomes than those without CDI. To improve patient outcome, clinicians should have a high index of suspicion for CDI in all IBD patients presenting with a disease flare in order to rapidly establish diagnosis and prompt treatment of infection (Trifan et al., 2014).

Last but not least, we must be aware that recurrent CDI recurrent CDI is more common in IBD patients compared to patients without IBD (Khanna, 2020), and each recurrence is increasingly difficult to treat, even in non-IBD patients.

The association and effects of CDI in patients with IBD remains a challenging topic of debate.

3.3.2. Incidence and risk factors of *Clostridium difficile* infection in patients with inflammatory bowel disease

Background & aim. For several years there has been a concern there about the increase in both incidence and severity of CDI worldwide (Khanna and Pardi, 2010). Several studies demonstrated an increasing incidence of CDI in patients with underlying IBD, with a more severe course of the disease compared with the non-IBD population (Rodemann et al., 2007; Issa et al., 2007; Nguyen et al., 2008). The incidence of CDI among hospitalized IBD patients increased from 1% in 1998 to 3% in 2007 (Ananthakrishnan et al., 2011). Both UC and CD present high-risk for CDI, but patients with UC are more susceptible than those with CD (Jodorkovsky et al., 2010; Murthy et al., 2012).

Therefore, in the present study we aimed to evaluate the incidence and the risk factors associated with CDI in IBD patients admitted in a tertiary center.

Materials and methods. We conducted a prospective, case-control study on IBD patients admitted to the Iasi Institute of Gastroenterology and Hepatology between January 2012- July 2014.

Demographic data and clinical characteristics like comorbidities, previous antibiotic use, immunomodulators, acid suppression, length of hospital stay, gastrointestinal surgery and outcome were assessed. Patients with indeterminate colitis, other infectious colitis and sepsis were excluded.

Diagnosis of CDI. All patients with flare activity were tested using immuno-chromatography assays for toxins A and B. Patients who tested positive for *C. difficile* toxins A and B were identified (n=26) and matched with IBD patients with negative *C. difficile* toxins (n=52) hospitalized during the same period of time.

Statistical analysis. The statistical analysis was carried out using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Continuous data were expressed as mean +SD and categorical data as percentage. The Kolmogorov-Smirnov test was used to determine whether the continuous variables were normally distributed. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. The Student t test or Mann-Whitney U test were used to com- pare groups, with the chi-square test used for categorical variables. Risk factors for CDI in patients with IBD were determined using logistic regression for univariate analysis. A p value of less than 0.05 was considered statistically significant.

Results. Incidence of CDI. Baseline demographic, clinical, and laboratory characteristics of the study population are outlined in Table 3.I. There was no significant difference between the 2 study groups.

During the study period, a total of 78 patients were admitted with the diagnosis of IBD of whom 26 (33.3%) had concomitant CDI. Of the IBD patients, 43(55.1%) had UC, and 35(44.8%) had CD.

Table 3.I. Baseline characteristics of study population

Parameter	*C. difficile* n=26	Control n=52	p-Value
Age (yrs) (mean±SD)	50.96±18.16	44.60±16.57	0.126
Sex m/f	16/9	33/19	0.363*
UC/CD	21/5	22/30	**0.001***
Albumin (g/dl) (mean ±SD)	3.63±0.67	4.36±0.83	**0.002**
Hemoglobin (g/dl) (mean ±SD)	12.46±2.25	12.88±2.28	0.450
Platelets (x10^9/mmc) (mean ±SD)	351±16.88	350±15.33	0.966
ESR (mm/h) (mean ±SD)	34.22±17.69	32.02±33.63	0.777
CRP (mg/dl) (mean ±SD)	4.81±6.26	4.81±6.26	**0.045**

Abbreviations: UC, ulcerative colitis, CD, Crohn's disease, SD, Standard deviation, ESR, erythrocyte sedimentation rate, CRP, C-reactive protein. *test X^2

We assessed the annual incidence of CDI in patients with IBD and found that it increased from 2.01% in 2012 to 16% in 2014 (Figure 3.3). Likewise, UC patients were more susceptible to CDI than CD patients (Figure 3.4).

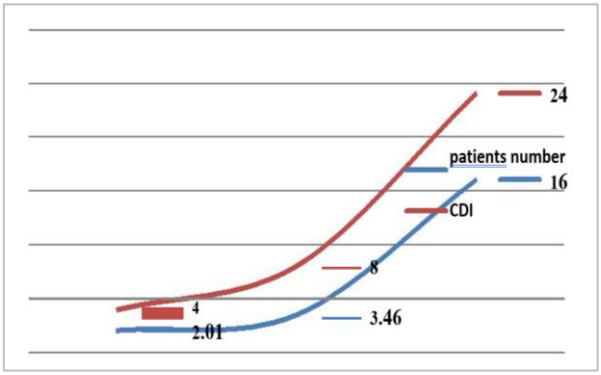


Fig.3.3. Annual incidence of CDI in patients with IBD

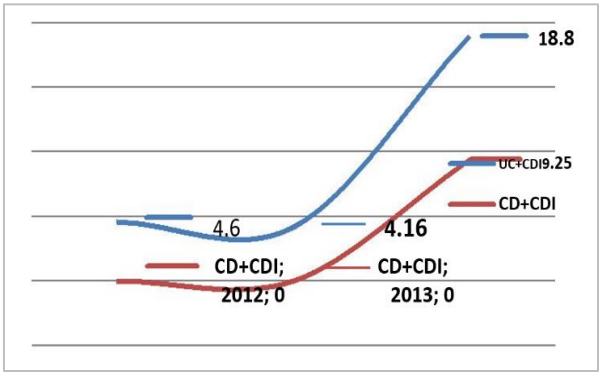


Fig. 3.4. Annual incidence of CDI in patients with UC and CD

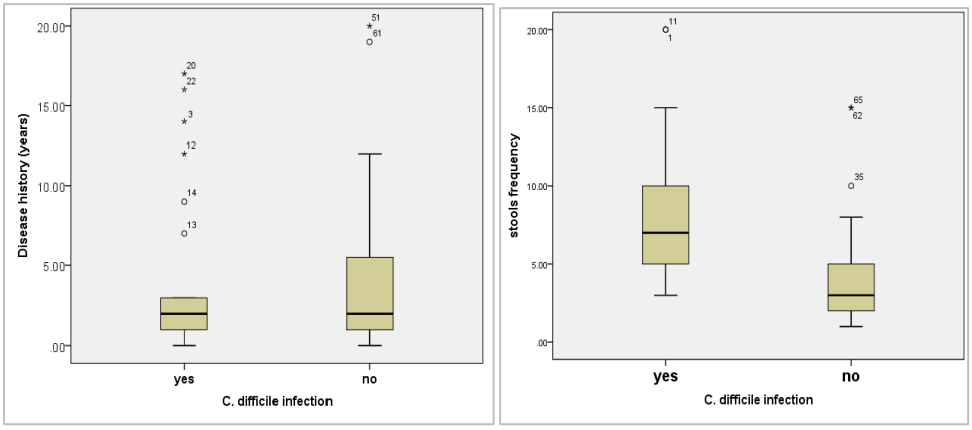
Risk factors for CDI. Antibiotic use prior to CDI was reported in 43 patients (55.1%), the most commonly used antibiotics being ciprofloxacin (36.1%) and ampicillin (19%) for urinary tract and upper respiratory tract infections. In our study, the length of hospital stay was not influenced by CDI (10.42±7.34 vs. 8.01±6.14, p=0.129) (Table 3.II).

Table 3.II. Risk factors for CDI

Parameter	OR	IC 95%	p-Value
ulcerative colitis	1.90	1.320-2.720	**0.001**
proton pump inhibitors use	1.57	1.133-2.032	**0.012**
antibiotic use	2.3	1.587-3.332	<**0.0001**
albumin <3g/dl	1.78	1.023-5.558	**0.038**

C. difficile infection was more common among patients with a recent diagnosis of IBD. Likewise, patients with an IBD flare- up were more susceptible to CDI (8.07±4.48 *vs.* 3.94±3.13, p<0.0001) (Figure 3.5). Cardiovascular co morbidities and malignancies did not influence the risk for CDI in our study population.

In our study, IBD patients with CDI were treated with metronidazole 250 mg orally four times daily for 10 days. In case of metronidazole failure vancomycin treatment was initiated. This was required in 2/26 (7.69%) patients. In this study the rate of CDI recurrence was 16%.



a. b.

Fig. 3.5. CDI in IBD patients; a - diagnosis of IBD, b - IBD flare-up

Discussion. A dramatic increase in CDI has been documented over the last two decades (Rodemann et al., 2007; Issa et al., 2007; Ananthakrishnan et al., 2008; Jen et al., 2011). Today, CDI is recognized as the main cause of infectious nosocomial diarrhea associated with increased morbidity, mortality and healthcare costs (Ricciardi et al., 2009).

Several studies have reported a significantly increased incidence of CDI among IBD patients, particularly in those with UC. Thus, a study using nationwide data showed that in US the incidence of CDI among hospitalized UC patients has doubled over a 7- year period (Nguyen et al., 2008). Likewise, a Belgian study reported a nearly four-fold increase in CDI in both IBD and non-IBD patients between 2000 and 2008 (Bossuyt et al., 2009). Our results showed that the incidence of CDI in IBD has increased during the study period for which data were available. Also, CDI is significantly more common in UC than in CD.

The classical risk factor for CDI is exposure to broad-spectrum antibiotics. Antibiotics disturb the normal intestinal flora, allowing *C. difficile* to proliferate (Ananthakrishnan et al., 2010). In this study, we found that previous antibiotic therapy and previous proton pump inhibitors treatment represent risk factors for CDI development in patients with IBD. Additionally, immunosuppressive and anti- TNF-α treatments were not risk factors for CDI.

Contrasting results have been reported regarding the length of hospital stay in IBD patients with CDI. Jodorkovsky et al. reported a similar mean length of hospital stay for UC patients with and without su perimposed CDI (11.7 *vs.* 11.0 days; p = 0.70), while Bossuyt et al. found a shorter stays in IBD patients with CDI (mean 15.2 days) as compared to non-IBD patients with *C. difficile* (mean 27.7 days) (<0.001) (Bossuyt et al., 2009; Jodorkovsky et al., 2010). The length of hospital stay is not influenced by the presence of CDI.

Our findings suggest that CDI is more frequent in patients who present with IBD flare-ups. Therefore, clinicians should maintain a high index of suspicion for CDI in IBD patients, especially in those with UC.

Recurrence of CDI is present in 10-30% of IBD patients (Ananthakrishnan et al, 2010). In this study the rate of CDI recurrence was 16%. This is in agreement with previous studies that reported similar findings.

A major limitation of our study may be the small number of patients and the fact that it was a single center study. This could have an influence on the conclusions regarding risk factors. Another limitation of this study is the absence of routine CDI testing in all patients with IBD as in other studies, and this could have influenced the annual incidence rate of CDI.

Conclusions. Our results show that the risk of CDI in IBD patients is extremely high and the incidence appears to be increasing at a faster rate. IBD patients who present with symptoms of a flare must be tested for *C. difficile* toxins. A rapid and adequate diagnosis and treatment may improve prognosis in IBD patients.

Our study was the first Romanian one in IBD patients with CDI and our data were cited by other studies (Bloomfield and Riley, 2016; Anderson A et al., 2017; Dalal and Allegretti, 2021).

We pointed out that the main limitations of the study were the small number of patients and the absence of routine CDI testing in each IBD with symptoms worsening; I emphasize once again that the exclusion of CDI is mandatory in every IBD patient with flare. Moreover, we also did not analyze the recurrence of CDI in IBD patients and the therapeutic aspects in these patients.

In the last two years we have had another challenge - how the severe acute respiratory syndrome coronavirus 2 (SARS CoV2) infection has influenced the clinical and therapeutic approach in IBD patients? Our data showed that the incidence of patients newly diagnosed with IBD increased from 2.95% in 2018 to 3.69% in 2020, a special situation being observed since the onset of the COVID-19 pandemic, when an increased number of patients newly diagnosed with a form of IBD was found in the total number of hospitalizations. The increased number of newly diagnosed cases of IBD since the onset of the COVID-19 pandemic is an assumption that requires further studies to demonstrate the impact of SARS CoV2 infection on the intestinal mucosa.

We aim to evaluate the epidemiological data of CDI in patients with IBD, on a larger group of patients and to analyze if and what has changed in recent years. We also want to analyze the recurrence rate of CDI, the predictive risk factors and develop a predictive risk model for it.

In the first wave of the SARS CoV2 pandemic, we analyzed the impact of the pandemic on the evolution of IBD on a small group of patients. Given the increased risk of CDI in patients with IBD, the increased incidence of CDI during the pandemic (at least in our patients), we wish to continue this study and to evaluate the outcomes of IBD patients with CDI during pandemic.

3.4. *CLOSTRIDIUM DIFFICILE* INFECTION IN PATIENTS WITH LIVER CIRRHOSIS

3.4.1. Introduction

Paralleling to increased incidence of CDI in the general population, there has been increased interest in CDI among patients with liver disease, particularly in those with liver cirrhosis (Bajaj et al., 2010; Ali et al., 2012).

Some studies have shown that CDI in patients with chronic liver disease is associated with high mortality and morbidity, high cost and worse outcomes (Bajaj et al., 2010; Trifan et al., 2015; Kierra et al., 2018).

Given the continuing growth in the incidence of CDI, it was expected that *C. difficile* would increasingly affect patients with liver cirrhosis, patients by definition with high risk of CDI. In addition to the general factors that favor bacterial infections in cirrhotic patients (previously discussed), there are several specific factors that predispose these patients to CDI, such as antibiotic use, hospitalization and frequent IPP use, multiple comorbidities, and immunosuppressant therapy (Bajaj et al., 2010).

Although it is known that the antibiotic use is one of the most important risk factor for CDI, cirrhotic patients need antibiotic therapy for either curative SBP or any other associated non-specific infection and most often, prophylactic purposes. Definitely, as long as prophylactic antibiotic therapy has proved to reduce mortality in patients with liver cirrhosis with refractory low protein ascites, history of SBP, or acute VB (Fernández et al., 2016; EASL, 2018; Ferrarese et al., 2021) it is impossible to give it up, even at the risk of developing CDI.

Cirrhotic patients frequent require admission and readmissions for acute decompensation of disease (HE, large ascites, gastrointestinal bleeding) and each hospitalization exposes these patients to CDI (Bajaj et al., 2019).

It seems quite difficult to reduce the incidence of CDI in cirrhotic patients by acting on these two risk factors; we cannot give up prophylactic antibiotic therapy (but we must

carefully evaluate the administration with curative visa, only when necessary), and unfortunately for the moment we cannot significantly reduce the admission/readmission rate of cirrhotic patients, (especially in our patients – some of them non-compliant, most of them with alcoholic liver disease).

Bajaj has specified that PPI use is a risk factor for CDI in cirrhotic patients (Bajaj et al., 2019). PPI are commonly used in patients with liver cirrhosis sometimes justified (gastroesophageal reflux disease, peptic ulcer disease) other times with doubtful indication (routine administration in variceal hemorrhages, healing of esophageal ulcer after endoscopic band ligation) (Lodato et al., 2008). 63% of patients receive unjustified PPI (Garcia-Tsao, 2011). As we previously mentioned PPI is a safe group of drugs, with great benefits when prescribed for well-established indications. Clinicians should be aware of the risk of CDI when prescribing PPI therapy, in patients with high risk especially in hospitalized patients on antibiotics, as cirrhotic patients often are (Trifan et al., 2013). Given that many prescriptions fall outside accepted indications it is possible that limiting the administration of PPI to cirrhotic patients only to those with a definite indication could reduce the incidence of CDI in these patients.

Like any other bacterial infection in cirrhotic patients, CDI is associated with increased risk of mortality, increased length of hospital stay, poor outcome (Bajaj et al., 2019), and may precipitate complications of cirrhosis, especially HE.

3.4.2. *Clostridium difficile* Infection in Hospitalized Cirrhotic Patients with Hepatic Encephalopathy

Background & aim. HE is the second most frequent major complication of liver cirrhosis following ascites, characterized by a wide spectrum of neurological or psychiatric symptoms, ranging from minimal disturbances in mental function to deep coma.

Paralleling the increased incidence of CDI in the general population, there has been increased interest for CDI in patients with liver disease, particularly in those with liver cirrhosis who are at high risk for CDI development because of their frequent and prolonged hospitalizations, the antibiotic treatment and proton pump inhibitor (PPI) use and the multiple comorbidities (Bajaj et al., 2010; Musa et al., 2010; Ali et al., 2012; Sundaram et al., 2014; Mittal et al., 2014). To our knowledge, there is only one study which evaluated the incidence of CDI in cirrhotic patients treated with rifaximin for HE, reporting the absence of this infection in this set of patients. Thus, our study is the first to assess the incidence of CDI in all hospitalized cirrhotic patients with HE, regardless of the therapy they received.

The aim of this study was to evaluate the incidence and risk factors for CDI in patients with liver cirrhosis hospitalized for an episode of HE in a tertiary-care center.

Patients and methods. This is a retrospective analysis of patients with liver cirrhosis who were admitted with an episode of HE, from January 1, 2012 to December 31, 2014, at the Institute of Gastroenterology and Hepatology of Iasi, a tertiary referral center for North-East Romania. The patient medical charts were reviewed, and demographic information including age, gender, prior hospitalizations, clinical and laboratory parameters, etiology of cirrhosis, therapy for HE, antibiotics and PPIs use were carefully searched, as well as the presence of HRS, SBP, gastrointestinal bleeding, and ascites. All patients developing diarrhea (≥3 watery stools/day) were tested for CDI. The results of stool samples for toxins A and B (enzyme immunoassay, EIA) were analyzed, and the presence of any or both toxins was defined as CDI. Each patient's stool was tested only once. Length of hospital stay and mortality during admission were also analyzed. Hepatic encephalopathy was classified according to the West Haven criteria.

Cirrhotic patients with HE and CDI (study group) were compared with those without CDI (control group). As this is a retrospective study, no institutional approval was required.

Statistical analysis. Statistical analysis was carried out using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables with normal distribution are expressed as mean ± SD, otherwise median and the quartiles are used (Q1,Q3). Categorical variables are expressed as absolute values and percentages. Quantitative variables with normal distribution were compared using the Student's *t* test, while for those with non-normal distribution, the Mann-Whitney test was used. The Chi square test (Fisher exact test for small expected frequencies) was applied for categorical data. Univariate analysis was performed for each recorded variable. Variables with P-value < 0.1 in univariate analysis were included in multivariate analysis (logistic regression). Odds ratio (OR) with 95% confidence interval (CI) was calculated for qualitative variables included in the logistic regression. P value of less than 0.05 was considered statistically significant.

Results. Between January 2012 and December 2014, 231 cirrhotic patients with HE were admitted, of whom 17 (7.3%) were diagnosed with CDI. The overall CDI incidence rate was 57.2 cases per 10,000 patient-days. In all the 17 patients, diarrhea occurred later than 72 hours after admission, and therefore, all cases of CDI were considered hospital-acquired. Demographics and clinical and laboratory characteristics of all patients as well as of those with and without CDI are shown in Table 3.III. The majority of patients were male (56.7%), mean age was 60.9±10.3 years, and the most frequent cause of cirrhosis was alcohol consumption (62.4%). Most patients had HE stage 2 (68.4%) or stage 3 (29.4%) and severe liver disease (Model for End-Stage Liver Disease (MELD) median score 15; Child-Pugh score 9.5). Approximately three quarters of the patients were on PPIs for unspecified reasons.

There was no significant difference in the gender distribution, etiology of cirrhosis, severity of liver disease (MELD score, Child-Pugh score), encephalopathy stage, presence of some complications of cirrhosis (ascites, SBP, upper gastrointestinal bleeding), as well as for most of the laboratory parameters between cirrhotics with HE with CDI and those without CDI (Table I). However, the patients with CDI were significantly older (62.8±10.8 years vs. 58.6±10.1 years, P=0.028), had significantly higher levels of blood creatinine (1.0 mg/dl vs. 0.83 mg/dl; P=0.046) and more frequent HRS (52.9% vs. 15.4%; P=0.01), but they had a similar number of hospitalizations in the previous three months (P= 0.779).

Table 3.III. Patient demographics, clinical and laboratory parameters of all patients, and according to study groups

Parameter	All patients n=231	Study group n=17	Control group n=214	p-Value
Gender, male/female (%)	130/101 (56.7/43.3)	11/6 (64.7/35.3)	119/95 (55.6/44.4)	0.467[C]
Age, years, mean±SD	60.9±10.3	62.8±10.8	58.6±10.1	0.028[T]
Etiology of cirrhosis, n (%)				0.552[F]
HCV	50 (21.6)	4 (23.5)	46 (21.5)	
HBV	37 (16.0)	4 (23.5)	33 (15.4)	
Alcohol	144 (62.4)	9 (53.0)	135 (63.1)	
Child-Pugh class A/B/C, n (%)	16/92/108 (7.4/42.6/50)	0/5/10 (0/33.3/66.7)	16/87/98 (8/43.2/48.8)	0.284[C]
Child-Pugh score, median (Q1/Q3)	9.5(8/12)	11(9/12)	9(8/12)	0.151[M]
MELD score, median (Q1/Q3)	15 (11/20)	13.5 (11.25/18.5)	15 (11/20)	0.901[M]
Prior hospitalization (last 3 months), median (Q1/O3)	1 (1/3)	1 (1/3.5)	1 (1/3)	0.779[M]
Creatinine (mg/dl), median (Q1/Q3)	0.84 (0.64/1.2)	1.0 (0.74/2.04)	0.83 (0.64/1.2)	0.046[M]
Albumin (g/l), median (Q1/Q3)	3 (2.56/3.5)	2.5 (2.3/3.4)	3 (2.56/3.5)	0.063[M]
Bilirubine (mg/dl), median (Q1/Q3)	3 (1.78/5.67)	3.42 (1.89/6.84)	2.98 (1.78/5.68)	0.670[M]
INR, median (Q1/Q3)	1.5 (1.29/1.75)	1.48 (1.23/1.89)	1.5 (1.3/1.75)	0.960[M]
Ascites, n (%)	181 (78.4)	15 (88.2)	166 (77.6)	0.539[F]
SBP, n (%)	25 (10.8)	4 (23.5)	21 (9.8)	0.096[F]
HRS, n (%)	42 (18.2)	9 (52.9)	33 (15.4)	0.01[F]
UGIB, n (%)	29 (12.6)	1 (5.9)	28(13.1)	0.703[F]

Parameter	All patients n=231	Study group n=17	Control group n=214	p-Value
Encephalopathy, n (%)				0.330[F]
stage I	4 (1.7)	0 (0)	4 (1.9)	
stage II	158 (68.4)	9 (52.9)	149 (69.6)	
stage III	68 (29.4)	8 (47.1)	60 (28.0)	
stage IV	1 (0.4)	0 (0)	1 (0.5)	
Antibiotic therapy, n (%)				
Rifaximin	219 (94.8)	16 (94.1)	203 (94.9)	0.990[F]
Other antibiotics	113 (48.9)	14 (82.4)	99 (46.3)	0.004[C]
Lactulose, n(%)	169 (73.2)	9 (52.9)	155 (71.4)	0.088[C]
PPIs therapy, n (%)	180 (77.9)	13 (76.5)	167 (78.0)	0.990[F]
Hospitalization days, median (Q1/Q3)	12 (8/17)	12 (8.5/17)	11 (8/12)	0.434[M]
Death during hospitalization, n (%)	36 (15.6)	3 (17.6)	33 (15.4)	0.734[F]

HBV: chronic hepatitis B virus; HCV: chronic hepatitis C virus; HRS: hepatorenal syndrome; INR: International Normalized Ratio; MELD: Model for End-Stage Liver Disease; PPIs: proton pump inhibitors; SBP: spontaneous bacterial peritonitis; UGIB: upper gastrointestinal bleeding. C Chi square test; F Fisher exact test; T *t* test (Student); M Mann-Whitney U test

During hospitalization, a large proportion of patients with and without CDI received rifaximin as therapy for HE (83.9%), and 50.2% were treated with quinolones or cephalosporines for SBP, urinary or respiratory tract infections, and skin infections. In addition to the patients diagnosed with CDI, 33 (14.2%) developed diarrhea during therapy for HE, but all of them proved negative for *Clostridium difficile* (*C. difficile*) after the stool analysis, and diarrhea resolved with antimotility agents. The therapy for HE consisted of lactulose (16.3%), rifaximin (21.2%), or a combination of lactulose and rifaximin (62.5%). Metronidazole 500 mg 3 times daily and vancomycin 125 mg four times daily were used for treating CDI in 6 (35.3%) and 11 (64.7%) patients, respectively. The mean duration of treatment for CDI was of 10 days.

The results of the univariate and multivariate logistic regression analyses are shown in Table 3.IV. Variables with P-value <0.1 in the univariate analysis were included in the multivariate logistic regression: age, antibiotic therapy, SBP, and HRS. Age over 65 years, antibiotic therapy and HRS remained significantly associated with the development of CDI in cirrhotic patients with HE.

Table 3.IV. Univariate and multivariate regression analysis of risk factors associated with *C. difficile* infection in cirrhotic patients with HE

Parameter	Univariate analysis			Multivariate analysis		
	OR	95%CI	p-Value	OR	95%CI	p-Value
Age >65 years	4.83	1.64-14.25	0.002	4.029	1.271-12.773	0.018
Antibiotic therapy other than rifaximin	5.42	1.51-19.41	0.004	3.917	1.011-15.174	0.048
SBP	2.82	0.845-9.460	0.096	1.754	0.453-6.784	0.416
HRS	6.17	2.220-17.147	0.001	4.059	1.387-11.879	0.011
Prior hospitalizations (≥2)	1.033	0.384-2.778	0.949	-	-	-
Lactulose	0.731	0.463-1.153	0.102	-	-	-
Rifaximin	0.992	0.877-1.122	0.896	-	-	-
Creatinine >1.2 mg/dl	1.176	0.396-3.486	0.778	-	-	-
UGIB	0.415	0.053-3.254	0.703	-	-	-
PPIs	0.915	0.285-2.937	0.990	-	-	-
Ascites	2.169	0.479-9.817	0.539	-	-	-

CI: confidence interval; HRS: hepatorenal syndrome; OR: odds ratio; PPIs: proton pump inhibitors; SBP: spontaneous bacterial peritonitis; UGIB: upper gastrointestinal bleeding

Discussions. Several studies have examined the incidence of CDI in adult hospitalized cirrhotic patients, and most of them reported higher rates of CDI in these patients than in the general population (Bajaj et al., 2010; Musa et al., 2010; Ali et al., 2012; Sundaram et al., 2014; Mittal et al., 2014). This is not surprising given the high-risk of cirrhotics for bacterial infections (Bajaj et al., 2012). The patients with liver cirrhosis have many of the well-known risk factors for CDI: frequent and prolonged hospitalizations, frequent antibiotics and PPIs use, and an immunocompromised system . In addition to the cirrhotic patients, there are other comparable chronically ill subgroups of patients including those with inflammatory bowel disease (ulcerative colitis, Crohn's disease) in whom an increased risk of CDI is well documented. It was found that cirrhotic patients with CDI had a higher mortality and higher hospital costs, and had a longer length of hospital stay than those without CDI.

Moreover, it was shown that CDI yields the highest fatality rate among infections that occur in hospitalized patients with cirrhosis (Bajaj et al., 2012). Given the high incidence of CDI and its negative impact on the outcome of hospitalized cirrhotic patients, a recently published study (Saab et al., 2015) suggested that all these patients should be screened for CDI; however, the model used in this study did not include the new therapy for CDI, and the results are in contrast with the current guidelines which do not recommend screening of asymptomatic patients.

There is currently a paucity of data on the incidence of CDI in hospitalized cirrhotics with HE, the second most frequent major complication of liver cirrhosis following ascites. To the best of our knowledge, there is only one retrospective study evaluating the incidence of CDI in cirrhotic patients who received rifaximin for the treatment of HE; none of the 211 patients developed CDI during rifaximin therapy (Neff et al., 2013). Another study, published as abstract (Patel et al., 2014) also assessing the efficacy of rifaximin in HE, reported no cases of CDI. Furthermore, a systematic review and meta-analysis reported that rifaximin has a beneficial effect on HE and none of the included trials found an increased risk of CDI (Kimer et al., 2014). It was suggested that CDI is absent or it occurs very rarely in cirrhotics with HE, probably due to the protective effect of lactulose (by reducing short chain fatty acids production and suppressing *C. difficile* growth) (Musa et al., 2010) and rifaximin (by reducing enteric ammonia- producing bacterial loads) (Bass, 2006).

The present study investigates the incidence of CDI in all hospitalized cirrhotic patients with HE, regardless of the therapy they received. We found that 7.3% of cirrhotics admitted for an episode of HE developed CDI. This finding contradicts the results of the two studies mentioned above (Neff et al., 2013; Patel et al., 2014) which reported the absence of CDI in patients treated with rifaximin for HE. The high incidence of CDI in our study could, at least partially, be explained by the systematic stool testing for toxins A and B by EIA in all cirrhotic patients hospitalized with HE who developed diarrhea during therapy.

In addition, all our patients with HE diagnosed with CDI had several additional risk factors for CDI, including the use of antibiotics, advanced age, and an immune compromised system secondary to liver cirrhosis (Neff et al., 2013). Rifaximin, unlike the other antibiotics used for the treatment of HE, appears to be effective, safe and well tolerated (Bass, et al, 2010; Mullen et al, 2011). Other studies suggested rifaximin to be effective in the prevention and treatment of the recurrent CDI (Neff et al., 2010; Garey et al., 2011).

We should also mention that, in Romania, rifaximin is often used alone or in combination with lactulose in the treatment of HE. Although most studies regarding the long-term therapy with rifaximin show that the risk for CDI is absent or negligible, it should, however, be underlined that such a risk does exist, as demonstrated by our study, and some other cases reported in literature. Bass et al (Bass et al., 2010) in a randomized, double-blind, placebo-controlled trial, demonstrated that rifaximin significantly reduced the risk for an episode of HE as compared to a placebo over a 6-month period, and reported that 2 out of 140

patients (1%) who had received rifaximin developed CDI during treatment (none of the placebo group), but both patients presented several risk factors for CDI. A number of other cases of CDI, detected in long-term rifaximin therapy, were also reported (Zullo et al., 2012; Mullen et al., 2014). Mullen et al. (Mullen et al., 2014) found 6 cases of CDI (0.015%) in a cohort study of patients who had received rifaximin for at least 24 months; infections occurred in high-risk patients. These rare cases of CDI reported during rifaximin therapy indicate that there is a risk for this infection, and the prescription of a long-term treatment with this antibiotic requires "a note of caution" (Zullo et al., 2013).

Diarrhea in cirrhotic patients with HE may represent an adverse effect of the therapy used for HE, mostly of lactulose (Bajaj et al., 2012) and very rarely of rifaximin. According to the study by Neff et al. (Neff et al.,2013) 8% of their patients developed diarrhea during treatment with rifaximin, while none was diagnosed with CDI. Another study reported that less than 5% of the patients developed diarrhea while receiving rifaximin for the treatment of HE.

Our study has some strengths and also has several limitations. Thus, it is the first study evaluating the incidence using internationally recognized CDI surveillance definitions (McDonald et al, 2007), and the risk factors of CDI among hospitalized cirrhotics patients with HE in a Romanian tertiary referral center.

However, as a retrospective, single center study it is more likely to produce bias: undertesting for *C. difficile*, underdiagnosed CDI in some cases, missing data. In addition, the study provides no information on the *C. difficile* strain.

Conclusions. The hospitalized cirrhotic patients with HE are at risk for CDI during therapy with lactulose/rifaximin. Clinicians should have a high index of suspicion for CDI when evaluating cirrhotic patients hospitalized with HE who develop diarrhea, in order to rapidly diagnose and treat this infection. Further studies on CDI incidence and impact in patients with liver cirrhosis hospitalized for HE are warranted.

Our study was retrospective but was the first one that evaluated the incidence (using internationally recognized CDI surveillance definitions) and the risk factors of CDI among hospitalized cirrhotic patients with HE in a Romanian tertiary referral center.

We didn't analyze CDI recurrence rate and although the incidence of CDI was low in our patients, it would be interesting to assess the recurrence of CDI in patients with liver cirrhosis, given the overall increased risk of CDI recurrence. The treatment of recurrence is often difficult, but I think it can be more difficult in cirrhotic patients, by definition immunocompromised patients, with high risk of infectious complications, who often require repeated hospitalizations and antibiotic therapy.

Although Saab S, et al. consider that hospitalized patients with cirrhosis should be screened for CDI (Saab et al., 2015) the current guidelines do not recommend screening of asymptomatic patients, as long as *C. difficile* is a commensal agent. Moreover, I should emphasize that in cirrhotic patients, many of them with lactulose treatment, 1-2 semi-consistent stools per day is normal and they do not require CDI testing.

It is very important that the selection of patients included in this proposed prospective study to be carefully considered according with case definition (watery diarrhea/toxic megacolon and a positive laboratory assay for *C. difficile* toxin A and/or B in stools, pseudomembranous colitis revealed by lower rectosigmoidoscopy).

3.5. *CLOSTRIDIUM DIFFICILE* INFECTION IN HOSPITALIZED OCTOGENARIAN PATIENTS

Background & aim. Over the past two decades there has been a dramatic worldwide increase in both the incidence and severity of *Clostridium difficile* infection (CDI) (Khanna and Pardi, 2010). The most important risk factors for CDI are the use of antibiotics, hospitalizations and advanced age (Huttunen et al., 2012; Marwick et al., 2013). Other

potential risk factors, such as immunosuppression, multiple comorbidities, chemotherapy, renal insufficiency, hypoalbuminemia, organ transplantation, use of PPI, and the emergence of a new hypervirulent and easily transmissible strain of the bacterium known as NAP1 in some North American and European areas, have been identified to explain the increased incidence of CDI (Blondeau, 2008; Loo et al, 2011; Morfin-Otero et al., 2016).

Older patients are unanimously recognized as a vulnerable, high-risk population for CDI because they often have multiple comorbidities, and are more likely to have frequent and prolonged hospitalizations and to receive broad-spectrum antibiotics, which disrupt intestinal microbiota (Strausbaugh et al., 2003; Laffan et al., 2006; Calfee, 2008; Szabó and Böröcz, 2015).

In addition, host defense against infections including *C. difficile* is altered in older patients by senescence of the immune mechanisms associated with aging (Shin, et al., 2016). Older adults (aged ≥65 years) have almost all the above-mentioned risk factors for CDI. Most of the studies evaluating CDI in older adults included patients aged between 65 and 80 years, and just a few have assessed CDI in the very elderly (i.e. ≥80 years) (Lessa et al., 2015; Ticinesi et al., 2015; Leibovitz et al., 2016; Leibovici-Weissman et al, 2016; Lee et al., 2016; Marshall et al., 2017;). Life expectancy has increased over the past years in most countries, and the number of older adults with a high risk for CDI is also increasing. Several studies reported that in older adults, not only are CDI rates significantly higher than in younger age groups, but also elderly patients are at increased risk of adverse outcomes including mortality (Patel et al., 2016; Kyne, 1999).

The aim of the present study was to evaluate the risk factors, clinical course, treatment and outcome of CDI in hospitalized octogenarian patients.

Methods. This was a retrospective analysis of patients aged ≥80 years diagnosed with CDI at two tertiary referral centers for northeast Romania from 1 January 2014 to 30 September 2016. The patients' medical charts were reviewed, and demographic information including age and sex, prior hospitalizations, clinical and laboratory parameters, and antibiotics and PPIs use in-hospital and 2 months before admission were carefully searched, as well as the area the patients lived in (urban, rural, care home residence). Comorbidities were recorded in all patients, and classified as cardiovascular, pulmonary, metabolic, chronic renal failure, neurological, neoplastic and digestive. The diagnosis of CDI was based on the presence of diarrhea (≥3 watery stools within 24 h) plus the presence of any or both *C. difficile* toxins (A or B) in stool samples. Hospital-acquired CDI was defined as a stool positive for *C. difficile* toxins obtained at least 72 h after hospital admission. Clinical features, treatment and outcome (length of hospital stay, surgery, mortality, recurrence of CDI) were carefully recorded. Data for patients with CDI (study group) were compared with those from the older (≥80 years) patients hospitalized during the study period (control group). Follow-up evaluation was carried out 30 days after completion of CDI therapy in the study group and 30 days after admission in the control group by reviewing medical charts and by directly contacting patients or their physicians.

As this was a retrospective study and completely anonymous information was collected from existing records, no institutional approval was required.

Statistical analysis. Continuous variables with normal distribution are expressed as mean SD, whereas categorical variables are expressed as percentages and absolute values. The χ^2-test was used for categorical data. Quantitative variables with normal distribution were compared using the Student's *t*-test, whereas for those with non-normal distribution, the Mann–Whitney test was used. Univariate and multivariate analysis (logistic regression) were carried out on a number of recorded variables, and odds ratio (OR) with 95% confidence interval (CI) was calculated for quantitative variables included in the logistic regression. The

level of statistical significance was defined as $P < 0.05$. Statistical analysis was carried out using the SPSS 20.0 software (SPSS, Chicago, IL, USA).

Results. During the study period, there were 286 patients aged ≥80 years hospitalized in our institutions, and among them 79 (27.6%) were diagnosed with CDI.

The patients with CDI had a mean age of 83.9±3.3 years (range 80–94 years) and 39 (49.3%) were men. The age and sex distribution were not significantly different between patients with and those without CDI (83.9±3.3 years *vs* 83.7±3.1 years, $P = 0.652$; men 49.3% *vs* 42.5%; $P = 0.297$, respectively). Patients' characteristics (demographics, comorbidities and laboratory parameters) are shown in Table 3.V.

Table 3.V. Characteristics of patients with and without *Clostridium difficile* infection

Variables	Patients with CDI (n = 79)	Patients without CDI (n = 207)	Significance (p-Value)*
Age (years) Mean ± SD	83.9 ± 3.3	83.7 ± 3.1	0.652
Sex - male, n (%)	39 (49.3)	88 (42.5)	0.297
Comorbidities, *n* (%)	76 (96.2)	183 (88.4)	0.044*
Cardiovascular disease			
Arterial hypertension, *n* (%)	48 (60.7)	89 (42.9)	0.007*
Atrial fibrillation, *n* (%)	17 (21.5)	32 (15.4)	0.224
Chronic cardiac failure, *n* (%)	32 (40.5)	41 (19.8)	<0.0001*
Pulmonary disease - Pneumonia, *n* (%)	6 (7.5)	22 (10.6)	0.440
Metabolic disease (DM), *n* (%)	13 (16.4)	21 (10.1)	0.130
Chronic kidney disease, *n* (%)	21 (26.5)	24 (11.5)	0.001*
Neurologic disease, *n* (%)	10 (12.6)	36 (17.3)	0.330
Neoplasia, *n* (%)	13 (16.4)	36 (17.3)	0.851
Digestive - Liver cirrhosis, *n* (%)	7 (8.8)	17 (8.21)	0.860
Laboratory			
Neutrophils, /mm^3 (mean ± SD)	12392.2 ± 873.1	6239.8 ± 218.5	<0.0001*
Creatinine, mg/dL (mean ± SD)	1.48 ± 1.3	1.72 ± 7.7	0.786
Hemoglobin, g/dL (mean ± SD)	11.1 ± 2.1	11.8 ± 2.2	0.007*
Hospitalizations in 2 months before admission, *n* (%)	43 (54.4)	71 (34.3)	0.002*
History of surgical interventions, *n* (%)	7 (8.8)	2 (0.9)	<0.0001*
Antibiotics use in 2 months before admission, n (%)	31 (39.2)	44 (21.2)	0.002*
Antibiotics use during hospitalization for infections other than CDI, *n* (%)	59 (74.6)	66 (31.8)	<0.0001*
PPI use in 2 months before admission, *n* (%)	34 (43.1)	76 (36.7)	0.326
PPI use during hospitalization, *n* (%)	45 (56.9)	105 (50.7)	0.345
Treatment			
Vancomycin, *n* (%)	8 (10.1)	0 (0)	NA
Metronidazole, *n* (%)	44 (55.6)	0 (0)	NA
Metronidazole + vancomycin, *n* (%)	27 (34.1)	0 (0)	NA
CDI outcome			
Length of hospital stay, days (mean ± SD)	15.3 ± 5.1	11.1 ± 4.3	<0.0001*
Surgery, *n* (%)	0 (0)	0 (0)	NA
Recurrence, *n* (%)	9 (11.3)	0 (0)	NA
Death during hospital stay, *n* (%)	0 (0)	0 (0)	NA

CDI, *Clostridium difficile* infection; DM, diabetes mellitus; PPI, proton pump inhibitors; SD, standard deviation; NA, not applicable. **P*-values are statistically significant

Most of the patients with and without CDI (90.6%) had at least one chronic underlying disease, and over half of them (55.6%) had two or more. The most frequent comorbidities were cardiovascular disease (47.9%) and neoplasia (17.1%). Patients diagnosed with CDI had more comorbidities than those without CDI (96.2% *vs* 88.4%, $P = 0.044$). Of the comorbidities, arterial hypertension, chronic cardiac failure and chronic kidney disease were significantly associated with CDI. Within the 2 months before admission, 114 (39.8%) of all

patients had at least one hospitalization for underlying disease during which 75 (26.2%) received antibiotics and nine (3.1%) had a history of surgical interventions. Patients with CDI had more prior hospitalizations compared with those with- out CDI: 43 (54.4%) versus 71 (34.3%), $P = 0.002$.

There were no significant differences between the two groups in the use of PPI before admission or during hospitalization, whereas antibiotics use was significantly higher within 2 months before admission or during hospitalization for infections other than CDI in patients with CDI. The most used antibiotics were cephalosporins (3rd generation), fluoroquinolones and carbapenems.

Laboratory parameters measured at the time of diagnosis for the patients with CDI and on admission for those without CDI, showed significantly higher neutrophil levels in patients with CDI (12 392.2 ±873.1 *vs* 6239.8 ± 218.5, $P < 0.0001$), and lower hemoglobin levels (11.1 ± 2.1 *vs* 11.8 ± 2.2, $P = 0.007$). Among the stool samples evaluated for *C. difficile* toxins, 57 (72.1%) were positive for both toxins, 18 (22.8%) had toxin B and four (5.06%) had only toxin A. The most common symptom in patients with CDI was watery diarrhea without blood, with a variable frequency between four and 14 times a day. Moderate, cramping abdominal pain was present in all patients.

On univariate analysis, prior hospitalizations, history of surgical interventions, antibiotic use 2 months before or during hospitalization and comorbidities were associated with CDI in octogenarian patients (Table 3.VI). On multivariate logistic regression analyses, previous 2 months hospitalizations (OR 10.231, 95% CI 1.769–58.965, $P = 0.009$), antibiotic use 2 months before admission (OR 12.596, 95% CI 1.024–15.494, $P = 0.048$), antibiotic treatment during hospitalization (OR 6.302, 95% CI 3.510–11.316, $P < 0.0001$), arterial hypertension (OR 11.228, 95% CI 1.917–65.783, $P = 0.007$), chronic kidney disease (OR 4.474, 95% CI 1.037–19.299, $P = 0.045$) and chronic cardiac failure (OR 7.328, 95% CI 2.068–25.967, $P = 0.002$) were independently associated with CDI.

Table 3.VI. Univariate and multivariate regression analyses of risk factors associated with *Clostridium difficile* infection in hospitalized octogenarian patients

	Univariate analysis			Multivariate analysis		
	OR	**95% CI**	**p-Value***	**OR**	**95% CI**	**p-Value***
Previous 2 months hospitalization	1.80	1.240–2.620	0.003*	10.231	1.769–58.965	0.009*
History of surgical interventions	6.18	3.637–10.518	<0.0001*	4.995	0.412–6.589	0.207
Previous 2 months antibiotic treatment	1.81	1.259–2.622	0.002*	12.596	1.024–15.494	0.048*
Antibiotic treatment during hospitalization	3.80	2.422–5.960	<0.0001*	6.302	3.510–11.316	<0.0001*
Comorbidities	1.08	1.019–1.162	0.029*	3.341	0.977–11.425	0.055
Arterial hypertension	1.41	1.115–1.791	0.011*	11.228	1.917–65.783	0.007*
Chronic kidney disease	2.35	1.393–3.972	0.002*	4.474	1.037–19.299	0.045*
Chronic cardiac failure	2.04	1.395–2.999	0.001*	7.328	2.068–25.967	0.002*
Diabetes mellitus	1.64	0.866–3.118	0.190	NA	NA	NA
Proton pump inhibitors during hospitalization	1.12	0.889–1.419	0.417	NA	NA	NA
Urinary tract infection	0.74	0.331–1.628	0.574	NA	NA	NA
Liver cirrhosis	1.07	0.465–2.502	0.860	NA	NA	NA
Chronic leukemia	2.62	0.540–12.711	0.241	NA	NA	NA
Pneumonia	0.71	0.301–1.697	0.583	NA	NA	NA

CI, confidence interval; OR, odds ratio; NA, not applicable. **P*-values are statistically significant

Within the first 24 h after diagnosis, all of the patients received antibiotic therapy for CDI: vancomycin was used in eight (10.1%) patients, metronidazole in 44 (55.7%), and in 27

patients (34.2%) with intolerance to metronidazole or unresponsiveness in the first 2–4 days, the therapy was switched to vancomycin. In six (7.6%) patients with CDI, the antibiotic treatment for other infections (two respiratory and three urinary tract infection, one spontaneous bacterial peritonitis) was continued. The mean CDI antibiotic treatment was 8.5 ±3.3 days. Patients with CDI had an increased length of hospital stay than those without CDI (15.3 ± 5.1 days *vs* 11.1 ±4.3 days, $P < 0.0001$). None of the infected patients had surgery, and nine (11.3%) had recurrence of infection during the follow-up period. There was no mortality in patients with CDI during hospitalization. Two patients died within 30 days after the CDI diagnosis, none related to CDI (one myocardial infarction, one stroke).

Discussion. Older age is a well-recognized risk factor for CDI. It has been shown that the average age of patients with CDI hospitalizations was 20 years older than the age of those with hospitalizations for other reasons (Lucado et al., 2006). The older population is particularly vulnerable, and studies reported CDI rates twofold higher in those aged ≥65 years, and sixfold higher in octogenarians compared with the younger age group (Castrillon et al., 2013; Keller and Surawicz, 2014).

The pathophysiology of the entire gastrointestinal tract is altered in these individuals as a result of atrophic gastritis, reduced gastric acid secretion, diminished bicarbonate and mucus secretion, and decreased colonic motility and prostaglandin production – all of which contribute to the development of CDI (Bhutto and Morley, 2008). One study reported that the annual rate of CDI-related hospitalizations per 100 000 individuals among those aged >85 years is higher than for all other age groups combined (Lucado et al., 2006). Because of the steady rise in life expectancy, the number of older patients at risk for CDI is expected to increase.

The present study evaluated the risk factors, clinical features, treatment and outcome of CDI in hospitalized octogenarian patients. Using a stepwise logistic regression model for multivariate analysis, we have found that antibiotic use within 2 months before admission and during hospitalization for treatment of other infections than CDI, and comorbidities such as arterial hypertension, chronic cardiac failure and chronic kidney disease were independent risk factors associated with CDI. There are several factors to which patients with heart failure are predisposed to CDI: advanced age, multiple comorbidities and frequent hospitalizations, all leading to higher rates of bacterial infections, and a larger use of broad-spectrum antibiotics that alter gut microbiota determining an overgrowth of pathogenic bacteria (Mamic et al., 2016).

Recently, secretion of urea into the gastrointestinal tract ("colorectal axis") has been associated with an altered gut microbiota characterized by a decrease in the num- ber of beneficial members of the gut microbiota (e.g. *Bifidobacteriaceae*) and an increase of potentially pathogenic microbiota (e.g. *Clostridium perfringens*) (Kortman et al., 2017).

Thus, the present results support the findings of other studies reporting the risk factors and outcome of CDI in octogenarians (Castrillon et al., 2013; Keller and Surawicz, 2014). This group of patients is at high risk for CDI because they often have multiple comorbidities, frequent and prolonged hospitalizations, and antibiotic treatments leading to altered intestinal microbiota. To our surprise, we found no increased risk of CDI in octogenarians on PPI treatment. A possible explanation is that many of these patients might have atrophic gastritis with low gastric acid output, and PPI cannot further lower gastric acid secretion, without any additional risk for CDI.

All of the present patients with CDI had a moderate form of disease, none requiring surgery and none died during hospitalization, findings in contrast to that reported in other studies, which found high mortality rates and high rates of severe forms of CDI in octogenarians (Hall et al., 2012). One possible explanation for our favorable outcomes might be the moderate disease, prompt diagnosis and treatment of infection. It should be under- lines

that it is rather difficult to establish a real causal link between death and CDI in very old patients with multiple comorbidities (Gilca et al., 2012). None of our octogenarians with CDI died during hospitalization, but two of them died within 30 days after the CDI diagnosis, none related to CDI (one myocardial infarction, one stroke).

There are no specific therapeutic recommendations for older adults with CDI; however, some authors recommend vancomycin as first-line therapy in octogenarians with severe infection (Mizusawa et al., 2015). As a general rule, treatment should be based on the severity of the CDI disease. In our study group, the patients had mild or moderate disease and received mostly metronidazole treatment for CDI. Recurrence of CDI occurred in nine (11.3%) of the present patients, all being relapses (manifested in <8 weeks after initial infection).

In the present study, the length of hospital stay was significantly longer for patients with CDI than for those without, a finding that supports those reported by other studies in older patients (Castrillon et al., 2013; Keller and Surawicz, 2014).

The present study had some strengths and also several limitations. Thus, it is one of the few multicenter case–control studies examining risk factors and out-come of CDI in very old patients in whom the controls were matched for age. The present study had several limitations, such as being a retrospective, relatively small sample size, and the lack of long-term follow up.

Conclusion. Hospitalized octogenarians with previous hospitalizations or recent antibiotic treatment, arterial hypertension, chronic cardiac failure or chronic kidney disease are at risk for CDI. Clinicians should have a high index of suspicion for CDI when evaluating hospitalized octogenarians who develop diarrhea in order to rapidly diagnose and treat this infection.

3.6. DID SARS-COV2 PANDEMIC CAUSED AN ENDEMIC *CLOSTRIDIUM DIFFICILE* INFECTION?

Background & aim. The SARS CoV2 pandemic remains a challenge for all clinicians. Given our concern and ongoing analysis of CDI infection, the SARS CoV2 pandemic is a challenge in this regard.

The 2020th has certainly been a challenging one for gastroenterologists; this pandemic year has profoundly altered medical practice, and has brought multiple challenges in approaching patients with digestive diseases given that many digestive and hepatic manifestations of SARS CoV2 infection, most often residual/post-infection, may alter the course of patients with digestive disorders (especially patients with inflammatory bowel disease, advanced liver disease, etc.). The increase in the number of CDI cases was expected given that patients with COVID-19 have numerous risk factors for it: most of them are elderly patients, with multiple comorbidities (some of them requiring immunosuppressive treatment), often with prolonged hospitalization (frequently in intensive care units), usually treated with antibiotics.

Diarrhea is one of the most relevant symptoms of gastrointestinal involvement in patients with COVID-19 infection with a prevalence varied from 11 to 17% (Ana et al., 2020; Malfertheiner et al., 2020). SARS-CoV-2 virus can actively infect and replicate in the gastrointestinal tract through the angiotensin-converting enzyme 2 receptors with secondary disrupt the normal intestinal flora, leading to gastrointestinal symptoms including diarrhea (Pan Let , 2020; Liang W et al., 2020; Granata et al., 2020; Chen et al., 2020; Ferreira et al., 2020; Laszkowska et al., 2021) Before the COVID-19 pandemic the most common cause of diarrhea (after excluding inflammation, organic intestinal lesions) was irritable bowel syndrome and functional disorders.

During COVID-19 pandemic, many patients received antibiotic treatment, sometimes with no clear indication or as primary prophylaxis for pneumonia (Granata et al., 2020).

Considering the risk factors for CDI should we expected to an increase incidence and recurrences of CDI during the COVID-19 pandemic period? The question is natural given that patients with SARS CoV-2 infection have numerous risk factors for CDI: they received broad-spectrum antibiotic treatment, were hospitalized, most of them were elderly, had multiple comorbidities or had immunocompromised status.

One study showed that 91% of COVID-19 patients received antibiotic treatment (Laszkowska et al., 2021) but generally over 70% of COVID-19 patients were treated with broad-spectrum antibiotics (mostly respiratory quinolones), in order to treat or to prevent bacterial co-infections and super-infections (Chen et al., 2020; Spigaglia, 2020; Aguila et al., 2020).

Although, an Italian retrospective study during the COVID-19 pandemic found a significant decrease in the incidence of healthcare-associated CDI in 2020 compared to the previous three years (explained by increased pandemic precautions), other data show that COVID 19 departments actually had a higher incidence of CDI compared to non-COVID-19 wards, but without statistically significant difference (Ponce-Alonso et al., 2019; Bentivegna et al., 2021).

The aim of our study was to assess the impact of the COVID-19 pandemic on the characteristics of CDI patients and to analyze the factors that influenced the incidence of CDI during the COVID-19 pandemic.

Materials and methods. *Study population*. We performed a prospective observational study including patients with CDI between March 2020 to December 2020. We have analyzed data from this period because from 1 March 2020, the Clinical Hospital for Infectious Disease Iasi was declared Covid unit, and as a result the Institute of Gastroenterology and Hepatology was designated the clinic that hospitalizes patients with CDI. The diagnosis of CDI was based on the presence of diarrhea (≥3 watery stools within 24 h) associated with any *C. difficile* toxins A or B (enzyme immunoassay, EIA) in stool samples (Lee et al., 2021).

Hospital-acquired CDI was defined as a stool positive for *C. difficile* toxins at least 72 h after hospital admission. Each patient's stool was tested only once. In all patients included, we collected demographic data (sex, age, residence), clinical and laboratory parameters, use of antibiotics, information regarding previous hospitalizations, comorbidities, associated medication, previously COVID-19 infection, treatment of CDI, and discharge. CDI data (first episode/relapse and relapse number), length of hospital stay, and mortality during admission were also analyzed. The treatment started with Vancomycin 125 mg every 6 hours and therapeutic response was defined as the absence of diarrhea after at least 72 h of treatment. We have excluded patients with other etiology of acute diarrhea.

The study was approved by the Local Medical Ethics Committee (No. 12 /2020/ March 15th, 2020). All the patients provided the written informed consent before study inclusion or further analysis.

Statistical analysis. Categorical variables were expressed as frequency and percentage. Continuous variables were expressed as mean plus/minus standard variation for normally distributed continuous data. All data were normally distributed. Groups were compared using $\chi 2$ test for categorical variables, and using independent *t* test or Mann-Whitney *U* test for continuous variables (depending on data distribution). Univariate analysis was performed for each recorded data. Variables with $P< 0.1$ in univariate analysis were included in multivariate analysis (logistic regression). Odds ratio (OR) with 95% confidence interval (CI) was calculated for qualitative variables included in the logistic regression. *P* value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

Results. A total of 3562 patients were admitted to our tertiary hospital during the study period, of whom 447 (12.5%) were diagnosed with CDI. Most of the patients were males (243

patients, 54.3%), mean age 59.7±10.8 years and had previous hospitalizations (266 patients, 59.5 %). Baseline characteristics of the patients included in the study are presented in Table 3.VII.

Table 3.VII. Baseline characteristics of the study groups

Parameter	Past history COVID-19 *n* = 76	Non-COVID-19 *n* = 371	p-Value
Age in yr, mean ± SD	62.6 ± 14.6	56.8 ± 17.6	0.007
Male	35 (46.1)	208 (56.1)	0.110
Country side	18 (23.7)	170 (45.8)	< 0.001
Hospitalization days, mean ± SD	8 (5)	9 (7)	0.094
Alcohol consumption	33 (43.4)	109 (29.4)	0.017
AB during hospitalization	29 (38.2)	154 (41.5)	0.588
Previous AB treatment	46 (60.5)	132 (35.5)	< 0.001
Comorbidities	65 (85.5)	348 (93.8)	0.013
Liver cirrhosis	17 (22.4)	158 (42.6)	0.001
IBD	3 (3.9)	31 (8.4)	0.187
DM	0	16 (4.3)	0.065
Malignancies	8 (10.5)	50 (13.5)	0.486
CKD	5 (6.6)	30 (8.1)	0.656
Previous hospitalizations	62 (81.6)	204 (54.9)	< 0.001
Recurrence	19 (25.0)	50 (13.1)	0.011
Leukocytes, mean ± SD	11320 (8843)	11560 (6650)	0.203
CRP, mean ± SD	2.53 (10.3)	2.52 (10.4)	0.103
Death	5 (6.6)	26 (7.0)	0.893

AB: antibiotics; CKD: chronic kidney disease; CRP: C-reactive protein; DM: diabetes mellitus; IBD: inflammatory bowel disease; SD: Standard deviation

Of all the patients included in the study 76 patients (17.0%) had history of COVID-19 infection. All the COVID-19 patients were diagnosed with healthcare-associated CDI. Nineteen patients (25%) had recurrent form of CDI. In all patients the treatment started with vancomycin 125 mg every 6 hours orally. In the group including patients with history of COVID-19 infection, in 26 patients (34.2%) the dose of vancomycin was increased to 250 mg every 6 hours for 10 days, 28 patients (36.8%) received vancomycin 500 mg every 6 hours as they did not response to the initial dose, and in 14 cases (18.4%) vancomycin enemas were added. Two patients in the COVID-19 group received fidaxomycin, as they were non-responders to maximal doses of vancomycin. Seventeen patients, from the COVID-group, with recurrent CDI received the tapering vancomycin regimen. Compared with the COVID-19 group, the majority of the patients with no history of COVID-19 infection and CDI (302 patients, 81.4%) responded to the conventional doses of vancomycin (125 mg every 6 hours, for 10 days) and no patients needed fidaxomycin.

There was no significant difference in gender and hospitalization days as well as for the inflammatory syndrome between patients with past history of COVID- 19 infection that developed CDI and those without history of COVID-19 (Table I). However, the patients with history of COVID-19 infection and CDI had a higher mean age (62.6±14.6 *vs.* 56.8±17.6, *P*=0.007), previous antibiotic treatment (60.5% *vs.* 35.5%, *P*<0.001) and hospitalizations (81.6% *vs.* 54.9%, *P*<0.001), were chronic alcohol consummators (43.4% *vs.* 29.4%, *P*=0.017) and more prone to recurrent disease (25% *vs.* 13.1%, *P*=0.011).

Thirty one patients (6.9%) died during hospitalization. The mortality rate was similar in both groups (6.6% *vs.* 7.0%, *P*=0.893).

The results of the univariate and multivariate regression analyses are shown in Table 3.VIII.

Table 3.VIII. Risk factors for CDI post COVID

Parameter	Univariate analysis			Multivariate analysis		
	OR	CI	p-Value	OR	CI	p-Value
Age > 60 yr	2.321	1.455-3.703	< 0.001	2.591	1.452-4.624	0.001
Urban area	1.935	1.273-2.940	0.001	2.330	1.286-4.221	0.005
Previous AB treatments	1.632	1.223-2.178	<0.001	1.909	1.083-3.365	0.025
Previous hospitalizations	2.444	1.503-3.947	< 0.001	2.509	1.263-4.986	0.009
Alcohol consumption	1.248	1.014-1.536	0.017	2.550	1.459-4.459	0.001

AB: antibiotics; CDI: *Clostridium difficile* infection; CI: confidence interval; OR: Odds ratio

The multivariate analysis demonstrated that age more than 60 years old (OR 2.59, 95% CI 1.452-4.624, *P*=0.001), urban area (OR 2.33, 95% CI 1.286-4.221, *P*=0.005), previous antibiotic treatment (OR 1.90, 95% CI 1.083-3.365, *P*=0.025), previous hospitalizations (OR 2.5, 95% CI 1.263-4.986, *P*=0.009) and chronic alcohol consumption (OR 2.55, 95% CI 1.459-4.459, *P*=0.001) were risk factors for CDI development in patients with history of COVID-19 infection.

Discussion. During the current conditions of the COVID-19 pandemic that appeared more than a year ago and considering the risk factors of CDI, we were entitled to expect a significant increase, and why not, even an endemic of the latter infection.

The increase in the number of CDI cases was expected given that patients with COVID-19 have numerous risk factors for it: most of them are elderly patients, with multiple comorbidities (some of them requiring immunosuppressive treatment), often with prolonged hospitalization (frequently in intensive care units), usually treated with antibiotics (Loo et al., 2011; Debast et al., 2014; Magill et al., 2014; Lessa et al., 2015).

Our results demonstrated that 12.5% of patients admitted to our tertiary hospital were diagnosed with CDI. More than half of our patients with CDI have had previous hospitalizations and 17% of them have been previously hospitalized for COVID-19 infection. We found that all the COVID-19 patients were diagnosed with healthcare-associated CDI. Our results are completely different from those of an Italian retrospective study during the COVID-19 pandemic that found a significant decrease in the incidence of healthcare-associated CDI in 2020 compared to the previous three years (Ponce-Alonso et al., 2019). The authors explained the decrease of CDI by increased pandemic precautions.

The growing number of CDI is not the only cause for concern about this infection. In the last years, one of the clinical challenges in patients with CDI has been patients with recurrent infection, often difficult to treat. Recurrent CDI is defined as an episode of CDI occurring within 8 weeks of a previous episode (Debast et al., 2014; Song and Kim, 2019) and it may be due to relapse of the previous CDI by the same strain or reinfection by a different strain (Tang-Feldman et al., 2003).

About 15-30% of CDI patients with initial response to antimicrobial treatment have a risk of recurrence of the infection and it is important to note that the risk of further recurrence significantly increases (Song and Kim, 2019). In our cohort nineteen patients (25%) had recurrent form of CDI.

There was no significant difference in gender and hospitalization days as well as for the inflammatory syndrome between patients with past history of COVID-19 infection that developed CDI and those without history of COVID-19 However, the patients with history of COVID-19 infection and CDI were elderly, from the urban area, had previous antibiotic use and were chronic alcohol consummators.

Although the majority of the literature on the epidemiologic features of *C. difficile* infection is based on association with antibiotic therapy and hospitalization settings (Huttunen

R et al., 2012; Marwick et al., 2013; Aguila et al., 2020) some other potential risk factors for CDI, such as advanced age, immunosuppression, comorbidities, chemotherapy, renal insufficiency, hypoalbuminemia, organ transplantation, use of proton pump inhibitors (PPI), have been identified to explain the increased incidence of CDI (Loo et al., 2011; Lessa et al., 2015; Trifan et al., 2018).

COVID-19 infection may be present as acute diarrhea and abdominal pain. Even in these conditions with symptoms suggestive for COVID-19 infection, testing for *C. difficile* must be done every time, because patients with SARS CoV-2 infection are patients at high risk for CDI.

Although CDI can affect individuals of all ages, the elderly are recognized at high-risk for this infection (Trifan et al., 2018; Aguila et al., 2020). Older patients represent a vulnerable population for CDI because they often have multiple comorbidities, have frequent and prolonged hospitalizations, received broad-spectrum antibiotics and the host defense against infections is altered. At the same time, so far, the most affected by COVID-19 infection have been elderly patients, patients who usually had severe forms of the disease and who were frequently treated with antibiotics.

Sandhu et al. collected data of several studies regarding on concomitant antibiotic use in patients with COVID-19 in USA (Sandhu et al., 2020). Most of these patients received empiric antibacterial therapy with either moxifloxacin, cefoperazone, or azithromycin (Cox et al., 2020), antibiotics known to be strongly associated with CDI and authors report that CDI cases was a co-occurrence or sequel of overuse of antibiotics in COVID-19 patients (Brown et al., 2013; Sandhu et al., 2020; Cox et al., 2020; Huttner et al., 2020).

We found that chronic alcohol consumption was a risk factor for CDI after COVID-19 infection. Chronic alcohol consumption influence gut microbiota, decreasing the bacterial diversity and increasing intestinal permeability and systemic inflammation (Meroni et al., 2019).

No references to the increased risk of CDI infection in chronic alcohol users have been found but we have at least two explanations for our result: almost 40% of hospitalized patients were patients with liver cirrhosis whose main etiology was alcoholic one but at the same time there are numerous data that show that in the pandemic alcohol consumption has increased worldwide, sometimes even worrying (Eurocare, 2020).

Our study has some strengths and several limitations. This is the first prospective study that characterized CDI after SARS-CoV-2 infection. The identification of risk factors for CDI after COVID-19 highlights the importance of recognizing vulnerable groups, such as the elderly population and patients who consume alcohol. The limitations of our study are represented by the small sample of cases and the fact that our data came from a single-center care unit without information on the *C. difficile* strains. We do not yet have a definite explanation for the fact that patients with CDI after COVID-19 require higher doses of vancomycin.

Conclusion. We observed that patients with a history of COVID-19 and CDI were from an urban area, had a higher mean age, had previous antibiotic treatments and hospitalizations, were chronic alcohol consumers, and were more prone to recurrent disease. Also, escalating the doses of vancomycin to obtain the therapeutic effect was another feature of the patients studied. In these patients, the antibiotic treatment for COVID-19 should be personalized in order to diminish the risk of CDI.

A few more clarifications can be added; the patients in the study group were especially from the first wave of the pandemic and it is well known that at that time most infected

patients were elderly patients or young patients with multiple comorbidities who often developed severe forms of the disease, with constant administration of antibiotics and assistance in intensive care units. In the following waves of the pandemic, an increasing number of infections were registered in younger patients; at the same time, the forms of the disease were milder and the need for intensive care admission was reduced. Given these aspects, we intend to analyze as soon as possible the trend of CDI evolution in the SARS CoV2 pandemic, as well as the epidemiological aspects of CDI recurrence.

Synthesis of scientific contributions
Awareness - Our clinical case is the first published in Romania and it was an alarm sign regarding CDI - CDI is underdiagnosed, either because of low levels of awareness for CDI among clinicians or missdiagnosed
IBD - The first data in Romanian regarding the risk of CDI in IBD patients - IBD patients who have symptoms of a flare must be tested for *C. difficile;* rapid and adequate diagnosis and treatment may improve prognosis in IBD patients with associated CDI.
Liver cirrhosis - the retrospective study was the first one that evaluated the incidence and the risk factors of CDI among hospitalized cirrhotics patients with HE in a Romanian tertiary referral center. - clinicians should have a high index of suspicion for CDI in patients with HE who develop diarrhea in order to rapidly diagnose and treat this infection.
SARS CoV2 infection - the study regarding CDI in pandemic times (the first prospective one) showed that the identification of risk factors for CDI after COVID-19 infection highlights the importance of recognizing vulnerable groups. - Vancomycin dose escalation is often required in CDI patients with post-infection COVID-19 to obtain a therapeutic response.

SECTION II

FORTHCOMING PROJECTS AND DEVELOPMENT IN MY ACADEMIC CAREER

As before, the research activity will be closely related to the medical training and the patients' needs will dictate the research objectives. I am a university teacher and this title involves scientific and research activity but first of all I am a doctor, dedicated to the human being, the patient to whom my main concern, according to the oath taken is "first, do not harm". My medical and scientific activity has had and will have as its main subject the patient, especially the one with advanced chronic liver disease to whom we try to offer years or at least days of life.

Research over the past decade has made substantial progress in deciphering the pathophysiology of LC and improving the survival of cirrhotic patients. LC is now a redefined disease and we hope to be able to influence its natural history as much as possible.

For these reasons the direction of future research will focus on chronic liver disease, especially advanced disease with all its complications. I will still be open as before to collaboration with all colleagues involved in other studies/research projects.

I intend, on the one hand to continue and develop some of the previous research topics but at the same time I want to address other topics. The main purpose of these projects is to find answers to the question how can we improve the survival and quality of life of patients with LC.

The treatment of patients with decompensated LC involves the prevention and treatment of specific complications (De Franchis, et al, 2021). New disease-modifying agents are expected to be identified in the next few years through the systematic repurposing of existing drugs and the development of novel molecules (Caraceni et al, 2021). Until we have a miracle drug capable of improving the evolution of decompensated LC, we will try to repurpose the "old drugs" that have already been prescribed in LC.

I. OLD DRUGS - NEW CHALLENGE IN LIVER CIRRHOSIS

LC is the end stage of all chronic liver diseases regardless of etiology and is one of the leading causes of morbidity/mortality and health problem worldwide (Zatonski et al., 2010; Blachier, et al., 2013; Mokdad et al., 2014; Global Hepatitis Report 2017).

Mortality in LC is expected to increase by 2030 mainly due to the increasing prevalence of alcoholic liver disease and NAFLD (Estes et al., 2018). Cost-effective interventions are urgently needed to continue the fight against the prevention and treatment of viral hepatitis and the early diagnosis and prevention of alcohol-related LC or NAFLD.

It has been discovered in recent years that some drugs used for decades to treat LC complications have possible new therapeutic valences and even new indications.

I.1. Non-selective beta-blockers in liver cirrhosis - "*Keep your friends close, but your enemies closer"*

In 1980, Lebrec and colleagues showed in a randomized study that propranolol significantly lowered portal pressure in 18 patients with known cirrhosis of the liver and variceal bleeding (Lebrec et al., 1980).

For over 40 years, nonselective beta-blockers (NSBBs) have been the mainstay of therapy for portal hypertension in patients with LC, the major benefit being the prevention of primary and recurrent variceal hemorrhage (Ki Tae Yoon et al., 2020); NSBBs therapy alone is almost as effective as combination therapy in preventing bleeding with a tendency to decrease mortality (Garcia-Tsao, 2016). In addition, NSBBs reduces bacterial translocation and the risk of SBP, increases survival regardless of bleeding events and reduces the development of other cirrhosis complications (ascites, HE) (D'Amico et al., 2006; Senzolo et al., 2009; Rodrigues et al., 2020). Moreover, long-term treatment with NSBBs has improved survival without decompensation mainly by decreasing the onset of ascites and early use of NSBBs in patients with compensated cirrhosis has reduced the number of decompensation events (Ripoll et al., 2007).

Surprisingly, 30 years later after Lebrec's study, in 2010, a group called the "founding fathers" of NSBBs in portal hypertension published a study suggesting that this therapy has detrimental effects on survival in patients with cirrhosis and refractory ascites (Sersté et al., 2010). This observation has led to extensive discussions about the safety of NSBBs in patients with ascites, infection, or kidney damage that has had to answer whether NSBBs is favorable or harmful to the cirrhotic patient. These studies led to the "therapeutic window" hypothesis, which called into question the use of NSBBs in early cirrhosis without medium-large varicose veins and warned to avoid them in patients with end-stage liver disease and refractory ascites (Mookerjee and Mehta, 2013). This hypothesis proposed that the window for using NSBBs closes once the patient develops "further" decompensation, such as refractory ascites (Krag et al., 2012).

NSBBs can have harmful effects on cirrhotic patients through several potential mechanisms:

1. Decreased organ perfusion by lowering mean blood pressure, heart rate, and ventricular contractility;
2. may precipitate AKI (Sersté et al, 2015);
3. the onset of paracentesis-induced circulatory dysfunction after high-volume paracentesis; and
4. inducing a portal hypotensive effect that may increase the risk of PVT (Qi XS et al., 2014). Sersté's findings have been contradicted by subsequent studies that have shown that NSBBs has not been associated with increased mortality when analyzing ascites refractory to diuretic therapy (Leithead et al., 2015; Bossen et al,. 2015).

If we weigh the benefits and disadvantages of NSBBs, they are much more favorable than harmful to the patient with LC (Ki Tae Yoon et al., 2020).

Although used for over 40 years in the treatment of portal hypertension due to liver cirrhosis with undeniable beneficial effects NSBBs remains an open research topic as there are many new hypotheses awaiting an answer.

In the future, we intend to evaluate the following aspects:

I.1.1. NSBBs may improve mortality rates in ACLF?

ACLF is one of the complications of chronic liver disease with a very poor prognosis. Jalan R et al. were the first who described it in 2002 (Jalan and Williams, 2002). Moreau et al. conducted the first large multicenter prospective study in Europe in which the authors defined ACLF as acute decompensation of LC associated with organ failure and high short-term mortality rates. In addition, they investigated the relationship between NSBBs and systemic inflammation in patients with ACLF (Moreau et al., 2013). Some studies have found that systemic inflammation in patients with ACLF is closely associated with the severity of liver disease, complications related to portal hypertension, and poor survival (Jalan et al., 2014;

Cazzaniga et al., 2009). Cirrhotic patients are prone to develop elevated levels of bacteria and bacterial toxins in circulation, and the mechanism behind these changes may be the so-called "inflammatory phenotype" of cirrhosis (Ki Tae Yoon et al., 2020). Several studies have found that NSBBs may reduce systemic inflammation in patients with LC and may exert this effect by lowering mesenteric venous congestion and decreasing intestinal permeability (Madsen et al., 2013; Mookerjee et al., 2016; Moctezuma-Velazquez et al., 2017). Mookerjee et al. showed that NSBBs treatment was associated with lower degrees of ACLF and significantly more patients with ongoing NSBBs treatment had a favorable outcome (Mookerjee et al., 2016).

Moreau and colleagues found that the intensity of systemic inflammation (leukocytosis and elevated C-reactive protein levels) is parallel to the severity of ACLF (Moreau, 2013); Mookerjee RP et al. found that patients who discontinued NSBBs treatment had significantly higher mortality rates at 28 days and three months (Mookerjee, 2016).

We have expertise in ACLF but we have never looked at whether NSBBs influence the evolution and prognosis of patients with ACLF.

We aim to conduct a prospective study to analyze whether there are prognostic factors in the use of NSBBs for the evolution and prognosis of patients with ACLF. As an immediate goal, given the available database, we aim to analyze whether the reduction in inflammatory syndrome (estimated by CRP, white cell count) in ACLF patients treated with NSBBs is of statistical significance.

I.1.2. NSSBs and non- malignant portal vein thrombosis – is there any association?

PVT is a severe complication of LC due to further increase in portal venous pressure with the risk of variceal bleeding and worsening liver function (Englesbe et a.l, 2010; Werner et al., 2013;). Accumulation of thrombotic risk factors results in an incidence of PVT in cirrhotic patients ranging from 0.9 to 1.8%, increasing to 8.1% in hospitalized patients (Northup et al, 2006; Garcia-Fuster et al, 2008; Wu and Nguyen, 2012); the incidence of PVT is 1% in patients with compensated LC reaching 25% in those on the list of liver transplantation (Francoz et al., 2012). The pathophysiology of PVT is not well established and although there are currently very few predictive factors none of them are actually helpful from a clinical perspective.

In recent years, several studies have shown that NSBBs treatment in LC patients would increase the risk of PVT because it can significantly decrease the flow speed of the portal vein (Zampino et al., 2018; Anton et al., 2022). Moreover, Xu X et al. conducted a meta-analysis to determine whether NSBBs increased the risk of PVT (Xu et al., 2019), based on the assumption that NSBBs decreases portal venous pressure and blood flow and may cause turbulent or slow blood flow in the vessel and thus increases the risk of coagulation. The authors concluded that there is a significantly 4.6-fold higher risk of PVT in patients treated with NSBBs but they consider that the association simply reflects the nature of patients treated with NSBBs namely those with advanced LC.

One of our studies showed that thrombotic events in patients with cirrhosis are 2.5%, confirming that this population is not "anticoagulated" by definition. Cirrhotic patients with serum albumin level <3 mg/dl and MELD score> 13 have an increased risk of thrombotic events correlated with the severity of liver disease (Girleanu et al., 2012). Our old results are similar to the recent meta-analysis, namely that PVT is often associated with the severity of the liver disease.

Given these data, we will analyze whether in our patients with LC, the treatment with NSBBs influences the PVT occurrence. Furthermore, we aim to build a predictive model for the occurrence of PVT and to analyze the benefits and risks of NSBBs in patients at high risk of PVT.

I.2. Rifaximine – an antibiotic with new effects

Rifaximin is a non-absorbable rifamycin derivate. The first and foremost indication of rifaximin is EH. Numerous studies have shown its effectiveness in treating and preventing the recurrence of EH. The use of rifaximin it is associated with a lower risk of HE recurrence (Caraceni et al., 2021). Furthermore, there is clear evidence that rifaximin improves the survival of patients with LC (Bass et al., 2010; Bajaj et al., 2012).

In the last decade, the pathophysiology of decompensation of cirrhosis has profoundly changed. There are ample evidences that decompensated cirrhosis is characterized by sustained pro-inflammatory and pro-oxidant factors. The high blood levels of endotoxin and inflammatory factors, common in patients with LC, accelerated liver fibrosis and stimulated the production of vasodilator substances. These will lead to decreased systemic vascular resistance, activation of the sympathetic nervous system and renin-angiotensin-aldosterone system, and a hyperdynamic circulatory system (Betrapally et al,. 2017).

In the same time, experimental and clinical evidence suggest that rifaximin could have other beneficial effects on the course of cirrhosis (others that HE) especially through modulating the gut microbiome and the gut-liver axis (Caraceni et al., 2021). Some data suggest that rifaximin could interfere with many elements in the pathophysiological chain of liver cirrhosis decompensation, namely systemic inflammatory syndrome, portal hypertension, or bacterial infections.

Thus, rifaximin has been shown to have beneficial effects on the prevention of variceal bleeding, SBP, hepatorenal syndrome as well as in the treatment of refractory ascites (Vlachogiannakos et al., 2013; Hanafy and Hassaneen, 2016; Xin-Yue et al., 2020).

I.2.1. Does rifaximine reduce portal hypertension?

Confirmation of this effect would be an essential element in the evolution of patients with LC because we could expect a reduction in variceal bleeding, one of the most severe complications of cirrhotic patients. This hypothesis is based on the link between the portal pressure and systemic inflammation (Mookerjee, 2011). Vlachogiannakos et al. (Vlachogiannakos et al,. 2013) showed that rifaximin significantly lowered portal pressure after 4 weeks while another randomized study in LC with ascites showed no reduction in portal pressure after 4 weeks of rifaximin (Kimer et al., 2018). Kimer's results seem closer to reality. We say this considering that although the dynamic component of portal hypertension and markers of bacterial translocation can change relatively quickly, the regression of fibrosis is a slow process. In hepatitis C patients with a sustained virological response, the reduction of fibrosis stage occurs a few years after treatment, not after some weeks. So, it is unlikely that it will occur structurally after only four weeks of rifaximin treatment effects. If the portal pressure still drops after four weeks, this may happen through other mechanisms.

As an immediate objective, we aim to assess the risk of variceal bleeding in cirrhotic patients associated with Rifaximin treatment.

I.2.2. Dose rifaximine and NSBBs association require a lower NSBBs dose??

Another controlled study assessing the combination of rifaximin with propranolol showed a more significant impact on portal pressure reduction and necessitated a lower mean propranolol dose compared with propranolol alone (Lim et al., 2017). The authors showed that the addition of rifaximin showed an additive effect for reducing hepatic HVPG through more substantial prevention of bacterial translocation than standard propranolol monotherapy. Therefore, the rifaximin – propranolol association in clinically significant portal hypertension CSPH patients to reduce HVPG can be applied as a therapeutic option for managing portal hypertension complications and to reduce the NSBB dose. According to Lim YL conclusion,

we will analyze if the rifaximine and NSBBs association require a lower NSBBs dose in our patients.

In our clinical practice we do not monitor HVPG and evaluate the effectiveness of treatment with NSBBs depending on heart rate. Most of our patients with LC are constantly treated with rifaximin and NSBBs, but we have not yet considered whether rifaximin should reduce the dose of NSBBs. The association of rifaximin with NSBBs may sometimes explain the low tolerance of cirrhotic patients to NSBBs, those patients who tolerate minimal amounts of NSBBs without an apparent cause.

I.2.3. Rifaximin - Can it be a therapeutic option in NASH?

NAFLD is a frequent cause of chronic liver disease in our century, affecting one quarter of adults worldwide (Younossi et al., 2016). In 2030, the same time we want to eradicate the hepatitis C virus infection, it is estimated that the highest prevalence of NAFLD will be in Italy (29.5%) and the lowest (23.6%) in France (Younossi et al., 2019).

The pathogenesis of NASH is highly complex. The classic "two hits" theory considers that the first hit is fat accumulation in hepatocytes and the second hit is data of inflammatory cytokines, oxidative stress, or insulin resistance (Machado et al., 2020). This theory has been replaced by the "multiple parallel hits" hypothesis because many data are showing that the pathogenesis of NAFLD involves several hits: lipotoxicity, insulin resistance, adipocytokines, endotoxins, gut microbiome dysbiosis, oxidative stress, and innate immune responses by toll-like receptors that intercede liver inflammation (Abdel-Razika et al., 2018; Fujinaga et al., 2020).

NAFLD is characterized by an intestinal bacterial overgrowth, especially of Enterobacteriaceae, with the production of endotoxins and increased intestinal permeability. The overgrowth of small intestinal bacteria has been observed in patients with NAFLD. Increased intestinal permeability and altered GM composition enhance the higher translocation of lipopolysaccharides and endotoxins into the portal circulation. Many reports have observed that lipopolysaccharides induced cytokines play an essential role in NASH because they may stimulate liver fibrosis and liver injury (Abdel-Razika et al., 2018).

In an experimental study, it was shown that both angiotensin-II receptor blockers and rifaximin showed an antifibrotic effect on the NASH liver and the combination of angiotensin-II receptor blockers and rifaximin demonstrated a more significant antifibrotic effect compared to either of the agents alone (Fujinaga et al., 2020).

This research suggests that new therapeutic options for obesity and/or metabolic syndrome, therapies whose action is on specific gut flora, are open to research. We must emphasize that if these hypotheses are confirmed they will be a significant step forward as long as the treatment possibilities in NAFLD are currently limited. Some recent studies have shown that prebiotics and probiotics can be an alternative therapy in the treatment of NAFLD (Khan et al., 2021). Abdel-Razika et al., evaluated the efficacy of 6-month therapy of rifaximin in patients with NAFLD. They suggested that rifaximin therapy appears to be effective and safe in the evolution of NASH by reducing serum endotoxin and improving insulin resistance and proinflammatory cytokines (Abdel-Razika et al., 2018).

The data presented will be the subject of future research. We aim to assess whether rifaximin may influence the evolution of NAFLD using mainly the assessment of hepatic steatosis and fibrosis by vibration-controlled transient elastography VCTE with a controlled attenuation parameter.

Gut microbiota may play a significant role in the pathogenesis of NAFLD but despite promising results, high-quality randomized controlled trials are needed in future studies to confirm these hypotheses in real life and to understand better the role of gut microbiota in the emergence and progression of NAFLD.

II. NAFLD – transient elastography, new challenges

NAFLD has been so far one of the important research topics I want to further develop, from new perspectives. All my research topics have one thing in common - noninvasive evaluation in CLD. VCTE/CAP was recently shown to be very accurate for both steatosis and cirrhosis over the incremental stages of NAFLD (Eddowes et al., 2019).

II.1. Correlation of iron metabolism parameters in patients with NAFLD with liver fibrosis assessed by TE

In my doctoral dissertation we showed that liver iron deposits in patients with chronic liver disease (regardless of etiology) correlate with the degree of liver fibrosis. The results are based on the morpho pathological examination of liver biopsy products. Many studies have shown that hepatic iron overload, observed in many liver diseases, can accelerate the progression of liver fibrosis to LC and HCC (Mehta et al., 2019).

Serum ferritin in NAFLD, as in any CLD, frequently raises due to systemic inflammation. High iron reserves and serum ferritin have been assumed as an independent predictor of histologic involvement severity and progression of fibrosis (Kowdley et al., 2012; Mauss et al., 2013).

In the coming years NAFLD will probably be one of the main etiologies of CLD. Younossi et al., in a meta-analysis from 2016 showed a global prevalence of NAFLD of 25.2%, which is estimated to reach 33.5% by 2030 (Younossi et al., 2016).

Many of the patients with NAFLD/NASH have risk to progress to end-stage liver disease and the identification of patients with high risk of progression is mandatory (Chalasani et al., 2012). All guidelines recommend risk stratification using non-invasive tests for fibrosis in NASH patients considered at risk of progressive liver disease (Trasolini et al., 2021).

Elevated liver iron concentration, more than 35 μmol/g of dry weight (Pietrangelo et al., 2009) is observed in about 33% of adult NAFLD patients (Kowdley, 2016) and analogically with HHC it is suggested to be associated with increased LF (George et al., 1998). In some NAFLD/NASH patients, high liver iron concentration may not be associated with increased fibrosis, but this aspect is also present in haemochromatosis patients heterozygous for the compound C282Y/H63D HFE mutation (Bugianesi et al., 2004; Adams et al., 2006). In HHC the intracellular distribution of hemosiderin is a trigger for fibrosis, but it is possible that macrophagic iron overload (as in patients with NAFLD) may not have an initiating effect, but only a maintenance of fibrosis, initiated by other factors.

Kowdley et al. showed that high serum ferritin is associated with hepatic iron deposition and proved to be a useful marker in identifying NAFLD patients with increased risk for NASH and fibrosis (Kowdley et al., 2012). Serum ferritin is a potential biochemical marker of NAFLD severity that is widely available and relatively inexpensive.

Knovich et al. found that serum ferritin (iron storage molecule found in the liver, serum, bone marrow) has been correlated to increased histologic activity in NAFLD and fibrosis (Knovich et al., 2009), while in a recent study Trasolini et al. did not find a significant correlation between elevated ferritin and liver fibrosis as measured by TE (Trasolini et al., 2021).

Together with my colleagues, I intend to correlate the values of iron metabolism (sideremia, ferritin, transferrin saturation index) with LF evaluated by TE in patients with NAFLD.

II.2. NAFLD and low bone mineral density

Osteoporosis is a common systemic skeletal disease characterized by reduced bone strength and could predispose to an increased fracture risk. The causes of osteoporosis include increased age, low body mass index, low bone mineral density, vitamin D and calcium, smoking, alcohol, and other factors, e.g. Mortality and disability-adjusted life years attributable to low bone mineral density (BMD) have almost doubled over the past two decades worldwide (Sanchez-Riera et al., 2014).

Among the main pathophysiological factors linking NAFLD with decreased bone mass are vitamin D deficiency, growth hormone/insulin-like growth factor 1 axis disturbances, and chronic inflammation.

Several studies showed that patients with NAFLD are likely to have low BMD and increased risk of osteoporotic fractures (Lee et al., 2018).

Lee et al., and Mantovani et al., have demonstrated that patients with NAFLD are likely to have low BMD and increased risk of osteoporotic fractures, but the relationship remains controversial and the related mechanism is not completed (Lee et al., 2018; Mantovani et al., 2019).

In epidemiological studies, NAFLD was shown to be connected with diseases that are usually not dependent on obesity, such as sarcopenia and osteoporosis (Poggiogalle et al., 2017).

Zhai et al. found a negative association between BMD and NAFLD markers; NAFLD with advanced fibrosis was positively associated with the occurrence of spine fracture (Zhai et al., 2022). The same authors found that subjects with NAFLD-associated advanced fibrosis have a higher prevalence of fractures compared with controls, although the prevalence of osteopenia/osteoporosis was comparable between the groups.

Few studies demonstrated that the presence of significant liver fibrosis assessed via TE was independently associated with low BMD in NAFLD. Kim et al. showed that BMD was reduced in subjects with significant liver fibrosis (LS >7 kPa) and that the presence of significant liver fibrosis was correlated with low BMDs at all sites and significant liver fibrosis remained an independent determinant of low BMD at the femur among NAFLD patients (Kim et al., 2017).

Screening and surveillance of BMD in patients with NAFLD may be considered in future strategies and in guidelines for NAFLD management.

II.3. NAFLD and thyroid disease

NAFLD and relationships with endocrine diseases are real challenges for clinicians nowadays.

Several studies demonstrated that morbidity of NAFLD has an inverse association with thyroid hormone levels in the hypothyroid or euthyroid population (Jaruvongvanich et al., 2017). Hypothyroidism was highly prevalent in NAFLD patients, and was associated with increased NAFLD activity, but not with fibrosis and steatosis severity.

Some studies reported that the prevalence of NAFLD is 27.4–33.1% in euthyroidism population, and 35.7–36.3% in hypothyroidism population (Zelber-Sagi et al., 2006; Frith et al., 2009; Xu et al., 2011).

The mechanisms involved in the development and the progression of NAFLD in hypothyroidism patients are incompletely understood. The potential mechanisms include metabolic syndrome, dyslipidemia, insulin resistance, oxidative stress, and direct action of thyroid hormones on the hepatocytes (Lugari et al., 2018; Lonardo et al., 2019;). There are two lines of evidence care justify for investigating the association between hypothyroidism and NAFLD: the physiologic capacity of thyroid hormones to contrast the development of

some features of the metabolic syndrome and the thyroid hormones direct effects on the liver (Sinha et al., 2018; Mantovani et al., 2018).

Guo et al., in a meta-analysis including 26 observational studies, showed that patients with NAFLD had significantly higher TSH levels than those without NAFLD, and that hypothyroidism was significantly associated with an increased risk of NAFLD (Guo et al., 2018).

Given NAFLD is bidirectional and mutually associated with the metabolic syndrome (Mantovani et al., 2018) and that hypothyroidism is very often associated with features of the metabolic syndrome (Lonardo et al, 2019) a strong consideration for NAFLD should be given to patients with hypothyroidism, especially if they are overweight or obese (Posadas-Romero et al., 2014; Mantovani et al., 2018).

Hypothyroidism is a modifiable risk factor for NAFLD and, more, selective THR-β agonists have been identified as promising treatment of both NAFLD and dyslipidemia (Manka Pet al., 2019).

III. *CLOSTRIDIUM DIFFICILE* INFECTION

Regarding this topic some of the projects I proposed represent a continuation of those presented in the thesis but I also have new topics, which I have not analyzed so far.

III.1. Recurrence of *Clostridium difficile* infection

Recurrence of CDI (a relapse of CDI symptoms within 2 - 8 weeks of successful treatment of the initial episode) is a global problem in recent years leading to significant morbidity and increased healthcare costs (McDonald et al., 2018).

About 15-35% of CDI patients suffer from recurrent infections (Marsh et al., 2012) and most of the time it's a relapse of the same infection rather than a re-infection with a new strain (Figueroa et al., 2012). In Romania, 2 studies showed the same rate of recurrence of RDI, of 20% (Lupse et al., 2013; Laza et al., 2015). I intend to do some research on this topic because at first glance the recurrence rate seems to be higher than literature records and this could be explained by the fact that at the national level >75% of the tested *C. difficile* strains belonged to ribotype 027 (Popescu et al., 2018). Initial infection with the NAP1/027 epidemic clone was found to be a significant risk factor for relapse. Unfortunately, second and subsequent recurrences are even more common after the first recurrence and managing these patients is sometimes extremely difficult. (McFarland et al., 2002).

Which patients have a high infection risk?

All studies record that advanced age and antibiotic use are the most frequent factors for infection's recurrence. 2 studies have found hypoalbuminemia (albumin < 2.5 g/dL) as a significant risk factor and predictive factor for 90-day disease recurrence (Kim et al., 2010; Knafl et al., 2019). Regarding the use of PPIs, the study data are contradictory but a more recent study concluded that PPIs do not increase the risk of recurrent CDI (Brown et al., 2013; Dharbhamulla et al., 2019).

I propose to build a predictive model for the risk of recurrence and a closer analysis will be done in 2 groups of patients frequently hospitalized in our Institute and who have a significantly higher risk of CDI than other patients: patients with IBD and those with LC.

III.1.1. Recurrence of *Clostridium difficile* infection in IBD patients

Razik et al., showed in a retrospective cohort study that IBD patients were 33% more likely to experience a recurrence of CDI (Razik et al., 2016). The association of CDI with patients with IBD usually rises both diagnostic and treatment problems. Worsening of diarrhea due to flare of IBD can often be clinically difficult to differentiate from the presence of

superimposed CDI (Seghal et al., 2021). At the same time, the treatment of these patients is often a challenge, and it is increasingly difficult with each subsequent relapse.

III.1.2. Recurrence of *Clostridium difficile* infection in LC patients

The prevalence of CDI is higher in cirrhotic patients than those without LC. The associated CDI may have a major impact on the outcome of cirrhosis including higher mortality rates (Bajaj et al, 2010). Patients with LC have many risk factors for CDI - they are hospitalized patients, immunocompromised, frequently in need of antibiotic therapy, etc. In a recent study Phatharacharukul et al. found a rate of recurrence in patients with LC of 11.9%, exacerbated by a high comorbidity burden and the use of lactulose (Phatharacharukul et al, 2020). Recurrence of CDI in patients with LC is often difficult to treat and is a fairly common cause of readmission, decompensation of liver disease. In the last year, we have seen a recurrence of CDI in more and more patients undergoing evaluation for liver transplantation. The construction of a predictive model for the risk of recurrence in these patients and the correction of risk factors is mandatory to increase the chance of patients with LC to liver transplantation.

III.2. How the SARS CoV2 infection has influenced the clinical and therapeutic approach of CDI?

In the first wave of the SARS CoV2 pandemic we analyzed the impact of the pandemic on the evolution of IBD on a small group of patients. Given the increased risk of CDI in patients with IBD, the increased incidence of CDI during the pandemic (at least in our patients), we want to continue this study and to evaluate the outcomes of IBD patients with CDI during the next pandemic waves.

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My future research activity will focus mainly on the continuation of the study directions in which I have gained experience over the past 25 years but I want to develop other research topics with my colleagues.

The experience gained so far and the fact that I am a part of a special work team, with remarkable scientific results, the development opportunities offered by "Grigore T. Popa" University of Medicine and Pharmacy have all the arguments for a permanent academic and scientific development.

Grigore T. Popa University of Medicine and Pharmacy is partner in a unique research project in Romania - CENEMED - a multidisciplinary medical research-development platform in the NE region. The project will allow the development of a complex research center which also includes gastroenterology/hepatology. This is an excellent opportunity for new research projects aimed to increase survival and improve the quality of life of patients in the North- East region.

SECTION III

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