

**UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA  
DOCTORAL SCHOOL**

# **PHD THESIS**

**-ABSTRACT-**

## **ROLE OF IMMUNOLOGICAL MARKERS IN THE ASSESSMENT OF RENAL INJURY IN PATIENTS WITH LIVER CIRRHOSIS**

**Scientific Supervisor:**

**Prof.univ.dr. ION ROGOVEANU**

**PhD Student:**

**FIRU ȘTEFAN GEORGE**

**CRAIOVA**

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## **INTRODUCTION**

Chronic liver disease and its final stage, liver cirrhosis, occupy a prominent place among the causes of death worldwide. Renal dysfunction is one of the most common complications in patients with cirrhosis with poor prognosis in the short term. Among the causes of renal dysfunction in patients with cirrhosis, hepato-renal syndrome (HRS) occurs distinctly as a particular form of renal impairment. HRS diagnosis is mainly an exclusion, with many attempts to establish identification criteria for affected patients.

Acute kidney injury (AKI) is an emergency in nephrology, with the prognosis depending on early diagnosis and therapeutic intervention as soon as possible. Classical markers of renal dysfunction identify the reduction in renal function lately and have the disadvantage that they cannot determine the etiologic factor underlying renal involvement. For this reason, more and more research has been directed towards the discovery of AKI biomarkers like troponin used in cardiology. These have led to the development of promising molecules in well-defined clinical situations where the type and timing of kidney injury are known, but the reality of clinical practice is much more complicated, so studies validating these biomarkers have diminished the enthusiasm of specialists in the field and must be continued.

The overall objective of this research is to evaluate the role of immunological markers of renal impairment in patients with cirrhosis, the usefulness of these markers in diagnosis and prognosis in this particular patient population.

**KEYWORDS:** liver cirrhosis, acute renal injury, cystatin C, NGAL, IL-18.

## **THE STATE OF KNOWLEDGE**

In the general part of the thesis are presented the current concepts of liver cirrhosis and hepatorenal syndrome, problems of diagnosis of renal dysfunction in patients with liver cirrhosis and data about potential biomarkers that could facilitate the early diagnosis of AKI giving the possibility of successful therapeutic intervention for affected patients.

### **1. Liver Cirrhosis**

Liver cirrhosis is the ultimate, irreversible, stage of all liver diseases determined by a variety of injuries [Balabaud C, Bioulac-Sage, 2010]. Currently, four major causes of liver cirrhosis can be identified: alcoholic liver disease, chronic viral hepatitis C, chronic viral hepatitis B and non-alcoholic fatty liver disease. According to WHO reports, liver cirrhosis in Romania is ranked fourth in a list of causes of death, with approximately 11,000 deaths attributed to it in 2012 [Country statistics and global health estimates by WHO and UN partners].

The evolution of chronic liver injury to cirrhosis involves the onset of several processes including necro-inflammation, fibrogenesis, angiogenesis and parenchymal necrosis secondary to vascular occlusions, nodular regeneration. Fibrous tissue storage depends on the site of liver injury, but the final result is the quantitative and qualitative change of

extracellular matrix (ECM). The studies have attributed to the stellate hepatic cells the primary role in the deposition of excessive ECM, but recent research reveals secondary sources of fibrosis due to the involvement of other resident cells of the liver or from the general circulation. [Wells, 2008] The results of a previous study [Tache et al., 2015] support the multiple origins of myofibroblasts involved in ECM remodeling, which may come from several types of adult hepatic cells (hepatocytes, cholangiocytes, portal fibroblasts) following an epithelial-mesenchymal transition process.

In most cases, the diagnosis of liver cirrhosis is based on a detailed anamnesis and a careful clinical examination, in conjunction with various laboratory investigations and imaging techniques. Liver biopsy is the "gold standard" method for diagnosis in patients with hepatic impairment [Afdhal și Nunes, 2004], but the use of this method has progressively decreased with the development of new non-invasive methods (Fibroscan, Fibromax). [Foucher et al., 2006, Morra et a. 2007] The results of a previous study showed similar results for transient elastography and real-time elastography in the evaluation of fibrosis in patients with chronic hepatopathy. [Jieanu et al., 2015] The use of Fibromax for the evaluation of hepatic fibrosis is also supported by favorable outcomes in recent studies, the test proving an optimal alternative for liver biopsy. [Poynard et al., 2005, Ratziu et al., 2006]

## **2. Renal Involvement in Liver Cirrhosis**

The importance of renal impairment for the prognosis of patients with cirrhosis was recognized after the introduction of MELD score to assess the short-term mortality of these patients. Patients with liver cirrhosis have a number of features that make the differential diagnosis of renal dysfunction an important problem for clinicians. The new therapeutic strategies have improved the survival of patients with liver cirrhosis and kidney complications, but an early and accurate identification of renal impairment could further enhance their effectiveness. The lack of globally accepted diagnostic criteria has made it difficult to carry out precise epidemiological studies to determine the prevalence and incidence of renal impairment in patients with cirrhosis. Data from a series of studies reported a prevalence of acute renal dysfunction of approximately 19-20% in hospitalized patients with liver cirrhosis. [Garcia-Tsao et al., 2008]

The main three causes of AKI in patients with cirrhosis are: prerenal azotemia (~45% of cases), HRS (~ 23% of cases) and acute tubular necrosis (~ 32% of cases). [Garcia-Tsao et al., 2008] Positive diagnosis and etiologic classification of renal dysfunction are currently based on the identification of changes in conventional endogenous markers such as serum creatinine and urea concentration, further information being provided by the urine test. The glomerular filtration rate is the most important indicator of renal excretion with the central role in evaluating kidney function.

Diagnostic criteria for renal dysfunction in patients with cirrhosis have undergone several changes over the years. The ADQI-IAC Working Group proposed the use of the term

"hepato-renal impairment" to describe any renal dysfunction identified in the patients with liver cirrhosis who meets the criteria for AKI, CKD or HRS. [Piano et al., 2013] HRS is defined as renal failure occurring in patients with advanced chronic liver disease, or sometimes fulminant hepatitis, in the absence of other identifiable causes of renal dysfunction. Long ago this condition was considered irreversible, the only treatment accepted being kidney transplantation. Studies of the last decade have allowed a better understanding of HRS pathophysiology and have provided new insights into pharmacological treatment.

Paraclinical evaluation of HRS patients indicate the following abnormalities: increase in serum urea and serum creatinine, decrease in plasma and increase of urine osmolality, decrease of urine sodium concentration, increase in renin and plasma noradrenaline activities, hyponatraemia, hyperkalemia. [Wong et al., 1995] These patients also have paraclinical indications of hepatic impairment: hypoalbuminaemia, hyperbilirubinaemia, prolonged prothrombin time, etc. Although the discovery of HRS-specific biomarkers has been of particular interest for research, research has not yet been able to identify them.

### **3. Biomarkers of Renal Dysfunction**

Similar to acute myocardial infarction, AKI is an acute situation with a somber prognosis that requires early identification and therapeutic intervention. Thus, nephrology research has shown a particular interest in the identification of renal biomarkers of AKI, the last decade bringing to the forefront a series of promising molecules, including: Cystatin C (an endogenous marker of glomerular filtration [Nejat et al., 2010, Inker et al. 2012, De Souza et al., 2014]), Neutrophil Gelatinase-Associated Lipocalin - NGAL (biomarker for positive and differential diagnosis of AKI [Osterman et al., 2012, Haase et al., 2014, Firu et al., 2015]), Interleukin 18-IL-18 (proinflammatory cytokine produced by proximal tubules cells in various kidney disorders [Parikh et al., 2006, Edelstein et al., 2007]). Since the discovery of biomarkers, a number of clinical trials have evaluated their utility in early identification of AKI and its evolution, as well as in the differential diagnosis of types of renal dysfunction in various clinical scenarios, in the adult or pediatric population. [Osterman et al., 2012]

## **PERSONAL CONTRIBUTIONS**

### **4. Working Hypothesis and Objectives**

The overall objective of the research performed in this thesis was to evaluate new methods of diagnosis and evaluation of renal impairment in patients with cirrhosis useful in clinical practice, proposing the following specific objectives:

- Evaluation of the utility in the clinical practice of new definitions and diagnostic criteria for the impairment of renal function in patients with cirrhosis;
- The roles of new biomarkers for the early diagnosis of renal dysfunction in patients with cirrhosis.

### **5. General Methodology**

The investigations carried out were included in the following clinical studies:

1<sup>st</sup> Study. Evaluation of clinical and paraclinical parameters associated with the development of acute renal injury in patients with cirrhosis;

2<sup>nd</sup> Study. Role of serum cystatin C in evaluation of renal function in patients with liver cirrhosis;

3<sup>rd</sup> Study. Role of new biomarkers of renal impairment (NGAL, IL-18) in the positive and differential diagnosis of AKI in patients with cirrhosis.

We conducted an observational study, endorsed by the Commission of Ethics and Deontology of UMF Craiova, which included patients with liver cirrhosis with and without renal dysfunction, admitted to the Clinic of Gastroenterology of the County Emergency Clinical Hospital of Craiova.

Biological samples were collected to determine biochemical and haematological parameters for the evaluation of hepatic function and renal function, performed with an automated Abbott Architect biochemistry analyzer based on standardized methods. Measurement of immunological markers was performed in the Laboratory of Biochemistry of UMF Craiova. The determination of NGAL, cystatin C and interleukin 18 was performed by ELISA-based methods using a complete StatFax line (Awareness Technology Inc). For the statistical analysis of the data we used Microsoft Excel (Microsoft Corp., Redmond, WA, USA) along with the XLSTAT 2014 add-on for MS Excel (Addinsoft SARL, Paris, France) and IBM SPSS Statistics 20.0 Corporation, Armonk, NY, USA). The data obtained were recorded in Microsoft Excel files, then processed statistically to analyze the relationships between clinical and paraclinical data.

#### **6. 1<sup>st</sup> Study. Evaluation of Clinical and Paraclinical Parameters Associated with the Development of Acute Kidney Injury in Patients with Liver Cirrhosis**

The objectives of this study were: identification of factors associated with development of AKI in patients with cirrhosis; evaluation of the new AKI diagnostic criteria versus the traditional criterion; identification of HRS triggering factors in the studied group.

Of the total patient included (n = 55), 20 patients with AKI (36.4% of cases) were identified using the traditional diagnostic criterion (serum creatinine > 1.5 mg/dL), whereas applying diagnosis criteria from the AKIN classification, 22 patients with AKI (41% of cases) were identified. The other 33 patients constituted liver cirrhosis group without AKI.

In the AKI group of patients, 10 met AKIN criteria for AKI diagnosis at the time of admission, the rest developing this complication during hospitalization.

Epidemiological data from this study indicate a higher prevalence of cirrhosis in the rural area (62%), with male sex being more frequently affected (59%), especially among those aged 50-59 (n = 20), while alcohol consumption was identified as the predominant etiologic factor of hepatic impairment (n = 36). Statistically significant differences were noted between AKI and AKI-free cirrhotic patients in terms of hemoglobin, hematocrit, leucocyte count, prothrombin time and albuminemia. The presence of ascites, encephalopathy and

hyponatraemia was more commonly identified in patients with AKI. The trigger factor that predominantly contributed to the development of HRS was paracentesis, followed by spontaneous bacterial peritonitis and upper digestive haemorrhage.

### **7. 2<sup>nd</sup> Study. The role of serum cystatin C in the evaluation of renal function in patients with liver cirrhosis**

The objectives of the study were: evaluation of the role of cystatin C as an early predictor of AKI development in patients with liver cirrhosis; comparison of glomerular filtration rate using formulas based on serum creatinine and/or serum cystatin in this patient population.

Patients were divided into two batches according to the serum creatinine value at the time of collection of the biological samples using the thresholds of 1.1 mg/dL for females and 1.2 mg/dL for male gender as follows:

- Lot 1 - 36 subjects with normal serum creatinine values;
- Lot 2 - 19 patients with elevated serum creatinine.

The serum creatinine-based equations (MDRD and CKD-EPI serum creatinine) identified 19 patients with eGFR below 60 ml/min/1.73 m<sup>2</sup> (34.5% of the total group). The CKD-EPI mixed formula, using serum creatinine and Cys C, revealed 22 patients (40% of the total lot) with eGFR of less than 60 ml/min/1.73 m<sup>2</sup>, and with the cystatin C formula (CKD-EPI CysC) we observed the eGFR below the threshold value in 32 subjects (58.2% of the total group).

According to the results of the study, patients with liver cirrhosis and a normal serum creatinine value are at an increased risk of developing AKI if serum levels of cystatin C are exceeded above the threshold of 1.27 mg/L. This threshold identifies patients at risk for AKI with a sensitivity and specificity of 75% and 96.43%, respectively. For a closer monitoring of patients with liver cirrhosis and normal renal function, the ROC curve determined the value of 1.1 mg/L as the threshold for identifying patients with normal serum creatinine but at risk of developing AKI with sensitivity and a specificity of 100% and 71.43%.

### **8. 3<sup>rd</sup> Study. Role of new markers of renal impairment (NGAL, IL-18, albuminuria) in positive and differential diagnosis of acute renal injury in patients with cirrhosis**

The main objective of this study was to evaluate the role of new renal biomarkers in patients with liver cirrhosis and renal impairment.

To evaluate the usefulness of NGAL and IL-18 in the positive diagnosis of AKI in patients with liver cirrhosis, two groups of patients were formed:

- Lot I - 33 patients with liver cirrhosis without acute renal injury;
- Lot II - 19 patients with liver cirrhosis and acute renal injury.

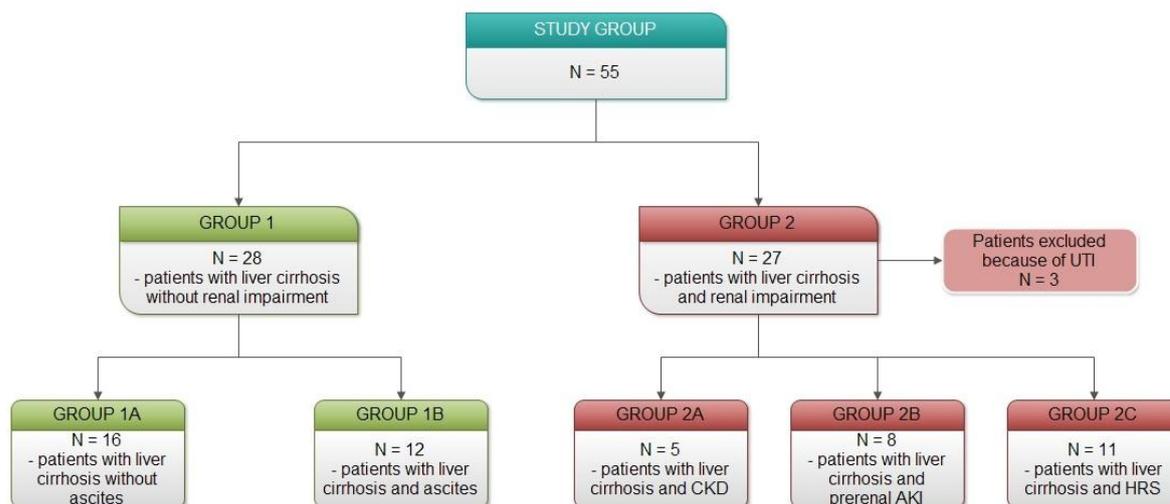
The Student t test revealed highly significant differences for serum and urinary NGAL levels, urinary IL-18 between the two groups. The statistical analysis allowed extraction of

the recommended threshold values for markers studied: 20 ng/mL for urinary NGAL, 125 ng/mL for serum NGAL and 20 µg/mL for urinary IL-18.

An attempt was made to establish a better method of detecting patients with AKI by combining the previous markers, proposing the model equation:

$$\text{Probability AKI} = 1/(1+\exp(-(-3.12974 + 0.17446 \cdot \text{UrineNGAL} - 0.02508 \cdot \text{IL-18 urine})))$$

To evaluate the usefulness of markers in the differential diagnosis of renal dysfunction in patients with cirrhosis, the total number of subjects enrolled in the study (n = 55) was divided into lots and sub-batches according to the following algorithm:



The ANOVA test revealed significant differences between the 5 groups for all the markers determined, the "post hoc" Fisher LSD test showing that the values for group 2C differed clearly from those for the other four groups.

To evaluate the role of the markers studied in the prognosis of patients with cirrhosis, we verified their correlation with MELD scores, widely used for the prediction of short-term mortality. All correlations are highly significant (p <0.001), except for the correlation between the MELD score and the urinary IL-18 level.

## 9. Conclusions

- The use of the new AKI diagnostic criteria could lead to a more effective identification of patients with cirrhosis that develops this complication;
- The development of AKI in patients with cirrhosis is associated with advanced disease;
- In patients with cirrhosis and normal serum creatinine, formulations based on cystatin C show a reduced eGFR relative to serum creatinine, which would justify the increased risk of these patients developing AKI
- Serum cystatin C serum may be useful in predicting the risk of developing AKI in patients with liver cirrhosis, the results of this study revealing two threshold values that can identify patients at risk with varying degrees of sensitivity and specificity;

- New kidney biomarkers (NGAL, IL-18) are useful in the positive diagnosis of AKI in patients with cirrhosis, but the presence of bacterial infections may be interfering factors in their determination.
- Higher levels of serum NGAL in patients with hepatorenal syndrome could be explained by the involvement of secondary mechanisms in the pathogenesis of this disease;
- NGAL values in urine and serum could be used as prognostic indicators in patients with cirrhosis, correlated with the MELD score, but further research is needed to confirm this hypothesis;
- This is the first study in Romania that aims to analyze the role of biomarkers mentioned above in assessing the renal impairment of patients with cirrhosis.

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