UNIVERSITY OF MEDICINE AND PHARMACY
"VICTOR BABEȘ" OF TIMISOARA
FACULTY OF MEDICINE
DEPARTMENT VII - INTERNAL MEDICINE II

MARC LUCIANA - ELENA

PHD THESIS

HEPATO-RENAL CROSSTALKS

A B S T R A C T

PHD Supervisor
PROF. SCHILLER ADALBERT, MD PhD

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THE GENERAL PART – CURRENT STATE OF KNOWLEAGE

Chronic kidney disease (CKD) was conceived of as an epidemiological concept with the main goal of early diagnosis of chronic kidney damage (regardless of the cause) that can lead to failing kidney function and, in turn, reduce the number of people who need renal replacement therapy (RRT).

CKD concept is based on a continuum that starts with risk factors (initiating factors) that act on the kidney for a long time, causing chronic, progressive damage that reduces the number of nephrons over time (i.e., reduces kidney function). In this continuum, the main goals are to aggressively stop modifiable risk factors, stop chronic damage early, and slow the rate of progression. So, early identifying of new possible risk factors and efficient treatment for slowing the progression of the disease seems to be mandatory.

Chronic kidney disease has many different initiating factors and among them, two disorders are leading, most frequently, to ESKD: diabetes mellitus (30% to 50%) and hypertension (27,2%). In 2021, 536.6 million (estimated 10,5 %) individuals worldwide were affected by diabetes and 9,2% of people in Europe has diabetes. Future projections suggests that by 2045, 783.2 million will develop diabetes. From 1990 to 2019, overall, the number of hypertensive people has doubled and it is estimated that by 2025 the prevalence will be over 60%. According to recent CDC data the CKD prevalence among hypertensive patients it is also increasing. Less frequent but not less important, in the genesis of CKD are involved glomerular diseases, chronic tubulointerstitial nephropathies, hereditary diseases and neoplasia or nephrotoxic drugs, traditional herbal remedies and HIV infections.

CKD is defined, according to KDIGO guidelines, as kidney damage and/or a glomerular filtration rate (GFR) of 60 mL/min/1.73 m (2) or less for three months or more. Kidney damage markers are represented by albuminuria levels, urine sediment or electrolyte or histology or structural abnormalities and also kidney transplantation. GFR is calculated using the CKD-Epi formula. According to KDIGO 2012, CKD is subdivided into 5 stages of disease evolution, based on GFR and the amount of albuminuria, with each stage having a corresponding risk category.

There are two types of risk factors for the onset of CKD: non-modifiable and modifiable. Non-modifiable risk factors include genetic predisposition, male gender, black race, low birth weight, older age, and loss of kidney mass from any cause, whereas modifiable risk factors include smoking, drug toxicity, diabetes mellitus, hypertension, obesity, autoimmune diseases, systemic infections, UTI, and kidney stones or obstruction. Also, AKI is recognized as an important risk factor for CKD initiation. From those mentioned risk factors – *diabetes mellitus, hypertension and obesity* proved to be the most important underlying CKD cause therefore it is mandatory that these patients receive active treatment in order to reduce de risk of developing CKD.

As knowledge progresses, new risk factors emerge and are evaluated as a result. Obstructive sleep apnea, fast heart rate, and periodontal disease are among the newly identified risk factors for CKD, but there are still inconsistent proofs. More recently, liver

diseases were associated with chronic kidney lesions and impairment. Non-alcoholic steatohepatitis (NASH) has a significant impact on the deterioration of kidney function. Non-alcoholic fatty liver disease (NAFLD) - the leading cause of chronic liver disease in Western adults is also recognized as a newly factor which is strongly associated with incidence and prevalence of CKD. The underlying mechanism of these findings is still unknown. The most plausible explanation for reduced kidney function is the confluence of cardiometabolic risk factors. The systemic release of pathogenic mediators from the fatty and inflamed liver, and their influence on kidney functions still remains an unclear territory. Also, chronic B and C hepatitis viral infections are linked to an increased risk of developing glomerular disease, and this link is most likely due to a causal relationship between the two. Not to mention that AKI is considered risk factor for CKD and also hepatorenal syndrome is severe liver disease induced AKI.

The connection between CKD and cancer is still not fully understood and seems to be a two-way relationship. Regarding the cancer risk in CKD patients, it is well known that oxidative stress and chronic inflammation are hallmarks in CKD and are also major risk factors for cancer development and reversely, malignancies may have a significant role in the onset of CKD both directly or cancer treatment related nephrotoxicity therefore cancers are accepted as CKD risk factors.

Once the process of chronic renal lesions starts, regardless of the underlying cause, CKD will progress more or less slowly whit irreversible nephron loss, up to end-stage kidney disease, and/or death at an earlier age. CKD progression is directly influenced by: higher levels of proteinuria (over 1g/24h), hypertension, poor glycemic control, smoking, obesity, dyslipidemia, the presence of cardiovascular disease, high dietary-protein intake, high dose of NSAID, decreased nephron number (related to age or other causes). CRIC study suggests that for slowing down the evolution of CKD, the right control of high blood pressure values and glycemic control is mandatory. Beside from RAS blockade (IECA, ARBs) which are the most effective in slowing down progression of the disease, other new treatments were developed. Endothelin 1 receptor antagonist (Atrasentan) and SGLT2 proved to be efficient in reducing albuminuria in diabetic kidney disease patients and by that slowing the progression of the disease. CKD progression is not always linear and predictable. Progressors, non-progressors and improvers have been described in some studies. Progressors are those patients already fulfilling stage G3b KDIGO criteria which develop high levels of albuminuria or microscopic hematuria; non-progressors are those patients in which the impaired renal function stays above stage G3b; improvers are those that from stage G3a have improved to stage 2 or even 1. When, where or why CKD progresses or remains stable or improves, is still a matter of debate. Syndrome of rapid onset end stage renal disease (SORO-ESKD) was also described. SORO-ESKD means unpredictable, unexpected, and fast deterioration of kidney function from CKD (eGFR >30ml/min) to permanent ESKD, often right after or 6–12 weeks after a fresh AKI episode. Factors that contribute to the SORO include young age, female gender, high diastolic blood pressure, elevated levels of albuminuria, high cholesterol levels, low serum albumin levels, and low hemoglobin values; still albuminuria was found to have the most significant impact on progression. SORO impacts mortality; SORO patients have a high mortality rate.

First medical description of ***fat accumulation in liver cells*** was registered in 1843 when the term of “fois gras” was first publish in a medical book. ***Hepatic steatosis*** became a more "visible" pathology after the introduction of liver biopsy, as a diagnostic method in clinical practice and represents a crucial contributor to the change of fatty liver disease from a disease of unknown origin into the most common and aggressively explored liver disease in present. After World War II, as a result of overnutrition, attention was ultimately drawn to NAFLD – a component of the metabolic syndrome. Non-alcoholic fatty liver disease (NAFLD), is a term, first described in the 80s and is now recognized as the major cause of CLD. The burden of illness is constantly increasing, the global prevalence in adults is estimated at 25.2% while in US according to NHANES, grows around 34% in the elderly with a trend of increasing numbers. Establish risk factors for developing NAFLD are diabetes mellitus, metabolic syndorme and obesity, but liver fat accumulation in normal-weight individuals, was also observed, especially in Asian populations and also the polycystic ovary syndrome and male sex has been suggested as a risk factor for NAFLD . In contrast, this disease appears to be less prevalent in the black race.

NAFLD is defined as presence of steatosis in more than 5% of hepatocytes, detected either on imaging or liver histology after the exclusion of secondary causes of liver fat accumulation (e.g., alcohol consumption, certain medication, other medical conditions). NAFLD has two main subtypes – non-alcoholic fatty liver (NAFL) and steatohepatitis (NASH). . NAFL is hepatic steatosis without hepatic cell injuries or hepatocyte inflammation, while NASH is steatosis and hepatocyte inflammation without fibrosis. In clinical practice, NAFLD is recognized as a pathological condition ranging from hepatic steatosis, which, if detected early, is usually considered reversible, to NASH which is an irreversible condition and leads to cirrhosis, liver failure, hepatocellular cancer and death. While the diagnostic of NAFLD is based mainly on clinical and imaging methods, for NASH, it is mandatory to perform a liver biopsy. Regarding screening of NAFLD the published data are scarce and contradictory. AASLD advises against screening, especially in high-risk populations, due to the limited availability of effective medication while European and Asian guidelines proposed that at-risk populations (obese, diabetic or individuals with metabolic syndrome) should undergo NAFLD screening. The progression of the disease is not linear and could be more dynamic than previously assumed, so noninvasive tools for diagnostic and staging and effective treatments are mandatory to be found.

Obesity-related NAFLD is predominantly caused by insulin-resistant fatty liver. Hepatocytes function of depositing lipids causes obesity-related steatosis. Hepatocytes have also an adipocyte-like function that increases fat storage in hepatic cells as triglycerides, leading steatosis onset. In response to initial inflammation, activated release proinflammatory cytokines and, as a consequence, starts the fibrotic process. If severe steatosis (SS) is left untreated, immune cells invade the liver and cause inflammation, which leads to NASH which is a non-reversible condition and progresses to cirrhosis. Studies showed that having obesity increases the risk of getting NAFLD by 3.5-fold, and that each additional unit of BMI increased the risk of NAFLD by 1.2-fold. Related to therapy, there are no targeted treatments available nowadays; weight loss and nutrition are the only tools of management available for NAFLD today.

T2DM increases the risk of NAFLD and predicts poor outcomes. Insulin resistance is the main link between T2DM and NAFLD. Excessive glucose substrate leading to free fatty acid buildup in hepatocytes stimulate hepatic lipogenesis. Proinflammatory cytokines, oxidative stress, and high triglyceride production, also cause inflammation, hepatocyte injury, and liver fibrosis. Important interactions between bile acids, modified bile acids, and FXR or TGR5 are regulating glucose and lipid homeostasis in NAFLD patients. Disturbances in hepatic lipid metabolism, inflammation of adipose tissue, and atypical sites of fat accumulation affect normal hepatic function and result in the accumulation of excess lipid droplets in hepatocytes. The presence of the two disorders not only impairs the patients' metabolic status, but also adds additional cardiovascular (CV) risk. Lifestyle modifications, including dietary interventions and weight loss, are standard recommendations for NAFLD management. There is no FDA-approved medication for NAFLD or NASH in patients with T2DM. Antihyperglycemic drugs such as GLP1-inhibitors and SGLT2-inhibitors have shown some benefits in reducing liver fat, but there is no evidence of a single effective medication. Pioglitazone is an off-label therapy that has been shown to improve liver histology in patients with biopsy-proven NASH with and without T2DM. Ongoing phase II and phase III RCTs are investigating the effectiveness of Obeticholic Acid, oltipraz, Aldafermin, Pegbelfermin, and other novel modulators of lipid metabolism and antifibrotic drugs. The ideal drug for NAFLD treatment would treat NAFLD, prevent T2DM and NAFLD-related CVD events.

Metabolic syndrome increases the risk of NAFLD due to a pro-inflammatory state and insulin resistance. Inflammatory markers like CRP, TNF-alpha, and IL-6 cause inflammatory cascades and disrupt insulin signaling in metabolic syndrome. CRP, the liver's main acute phase reactant, upregulates the NF-KB pathway and disrupts insulin signaling. Insulin resistance lowers insulin sensitivity and increases free fatty acids, which are stored in the liver and cause inflammation. 90% of NAFLD patients have one metabolic syndrome symptom, and 30% have three or more. Preventing obesity and diabetes, which are present in about half of NAFLD patients, can reduce MS-related NAFLD risk.

NAFLD was always an exclusion diagnosis. For more than four decades, all guidelines and research papers defined non-alcoholic fatty liver disease by excluding other liver pathologies and alcohol intake, which has no dose threshold. Many recent studies have cast doubt on long-held beliefs. The Framingham Heart Study found that alcohol use may cause steatosis in NAFLD patients, and other studies have linked alcohol consumption to liver disease progression. Other autors suggest that the link cannot be proven. Even the gut bacteria that produces alcohol (Klebsiella pneumoniae) may affect fatty liver disease. Another issue with this disease's name is that "non" implies no relationship between the disease and other factors (e.g., alcohol, metabolic syndrome, etc.) and that "non" means no progression, which is greatly misinterpreted. The word "non" limits discussion and hides metabolic syndrome's role in its development. This terminology also lowers self-esteem. Many patients only associate excessive drinking with liver disease, making it difficult to accept the diagnosis and make lifestyle changes. Thus, renaming is the first step in raising patients' awareness and understanding of their disease and emphasizing its risky relationship with metabolic syndrome. The change of terminology was indeed very challenging but in 2019, Eslam and colleagues called for consensus to address this need

and later published a consensual name for once called NAFLD. The chosen name was **METABOLIC ASSOCIATED FATTY LIVER DISEASE (MAFLD)**.

The definition of the new MAFLD concept - metabolic associated fatty liver disease, refers to a condition characterized by the presence of hepatic steatosis concomitant with type 2 diabetes mellitus (T2DM) or overweight/obesity or the presence of two or more metabolic abnormalities such as: waist circumference ≥102/88 cm in Caucasian (or ≥90/80 cm in Asian); blood pressure ≥130/85 mmHg or specific drug treatment; increased serum triglyceride levels (TGL) ≥150 mg/dl or specific drug treatment; low HDL-cholesterol > 2 mg/L; prediabetes; plasma C-reactive protein (CRP) >2.5; homeostatic model assessment (HOMA) score >2.5.

Excess body weight should be one of the three diagnosis criteria because of its pathological link to MAFLD and its ability to predict poor clinical outcomes. Obesity increases all-cause mortality. Obesity—even metabolically healthy obesity—increases MAFLD patients' risk of cardiometabolic complications and hepatic fibrosis . Obesity and metabolic syndrome may worsen MAFLD. Obesity and MAFLD are often seen together in clinical practice, so including obesity in the definition would help identify most patients through routine checkups, one of the goals of the term change.T2DM's effects on MAFLD onset and progression are not a matter of debate. As mentioned, NAFLD and T2DM are bidirectional. Having a new name—MAFLD—does not change the pathological relationship between those two disorders. Insulin resistance remains the main cause of fatty liver liver accumulation. The newly diagnosed disease's clinical duty of care is raised by the inclusion of at least two metabolic anomalies. Lean patients, who were previously ignored by clinicians, will now be evaluated for metabolic abnormalities. 6–20% of MAFLD patients are lean. However, lean people with metabolic dysregulations and MAFLD can develop severe steatosis and fibrosis, increasing their cardiovascular risk. The new definition also redefines the alcohol-fatty liver link. Since MAFLD is rising worldwide and alcohol intake has no safety "dose," it's easy to see why those two diseases often coexist. Alcoholic liver damage was always associated with ALD. The new terminology does not rule out MAFLD in alcohol consumers who meet the diagnostic criteria. These patients have double etiologies for liver damage, and the same diagnostic algorithm is used if they have other causes (e.g., HBV or HCV infection, HIV infection, autoimmune hepatitis, drug intake).

Currently available diagnostic tools for MAFLD are liver biopsy (invasive procedure), steatosis and fibrosis scores, as well as non-invasive ultrasonographic/computer tomography/MRI methods.

Liver biopsy, despite its inaccuracy and risks, has always been the "Gold standard" for NAFLD/MAFLD diagnosis. Liver biopsy increases the risk of death from 0.009% to 0.14%, evaluates only 1/50,000 of the liver parenchyma, and exposes patients to dangerous complications like peritoneal hemorrhage.

Also, steatosis and fibrosis ***scores*** were developed such as Fibrosis-4 (FIB-4), BARD, AST to Platelet Ratio (APRI), and NAFLD Fibrosis Scor but they did not help clinicians diagnose mild to moderate fibrosis. The FibroTest and Enhanced Liver Fibrosis (ELF) panel are more accurate, but they are expensive and require specialized biomarkers [89].

Imagistic methods were respresented first by *B-mode ultrasound* - the standard technique, but this classic technique have some important limitations in MAFLD patients: low sensitivity for mild steatosis; reduced diagnostic accuracy in obese patients; examiner-dependent method. So, in recent years *Quantitative ultrasound assessment of steatosis* - was created. Additional to traditional ultrasound, a number of techniques were developed in order to use the attenuation of ultrasound frequency for steatosis detection. They include: the controlled attenuation parameter (CAP), the ultrasound-guided attenuation parameter (UGAP), the attenuation coefficient (ATT), attenuation imaging (ATI). From all those new methods for evaluation , *CAP* has proven to be a reliable technique with a measurement failure rate between 0-24%. CAP can be measured by using two probes - standard probe (M probe), and an obesity-specific instrument (XL probe). Cut off values for CAP are published in an important metanalysis. *UGAP* has better histological steatosis grade diagnostic accuracy than CAP. *ATT* had no measurement failure and comparable histological steatosis grade diagnostic accuracy to CAP , but all these data come from trials involving a small number of patients. *MRI-PDFF* quantifies liver fat by measuring triglycerides in liver tissue. MRI-PDFF is the most accurate method for quantifying liver fat and diagnosing NAFLD steatosis but is not cost-effective method. Recent imaging methods for liver fibrosis diagnosis and staging have been developed - *VCTE, MRE, and shear wave elastography(SWE)*, but *MRE* outperforms SWE and FibroScan in obesity and cirrhosis. Due to high cost, long examination time, and limited availability, MRE is not routinely available in all clinical practice settings.

HEPATORENAL CROSSTALK was the aim of this thesis. The term ***c rosstalk***, borrowed from electronics, describes signal transduction pathway components' transmembrane or intracellular interactions. Disease-related "*organ crosstalk*" describes how dysregulations in one organ (e.g., liver) can affect another (e.g., kidney).The kidney has a complex, bidirectional relationship with other organs, according to decades of research. Cardiorenal syndrome (CRS), studied since 1836, is the best example of organ-to-organ crosstalk that could mean "dangerous liaison" in the kidney-heart relationship. We may assert that the organs of the human body have their own language – expressed by using signaling pathways and are intertwined like dominoes, suffering and benefiting from one another. This chapter of the thesis will analyze in detail, **the *LIVER – KIDNEY CROSSTALK*.**

Hepatorenal syndrome (HRS) was the first liver-AKI disease association. AKI in HRS is characterized by renal vasoconstriction in severe liver disease patients before renal impairment is clinically apparent. The pathopsychological complex pathway that causes HRS begins with arterial vasodilatation caused by changes in the cirrhotic hepatic parenchyma associated with liver vascular neoformation due to elevated proangiogenic factors. Secondary, systemic vascular resistance and decreased effective blood volume is installed, which activates RAAS and SNS via baroreceptors from the carotid and aortic arch to release vasopressin. Renal blood flow and GFR drop significantly. The kidneys should release lots of prostaglandins to prevent intrarenal vasoconstriction and maintain renal blood flow. The excretion rates of prostaglandins and cyclooxygenase (a kidney response to liver impairment) in the medullar cortex do not suggest this mechanism is active in HRS. HRS

development relies on prostaglandin synthesis reduction, contrary to our expectations. Severe renal vasoconstriction may reduce renal blood flow. HRS type 1 and 2 involve this pathologic mechanism. Type 1 HRS changes install faster than type 2 HRS.

Beside HRS, AKI's knock-out effects on neighboring organs like the liver are caused by *ischemia and ischemia-reperfusion.* Pro-inflammatory and pro-apoptotic cascades are both fueled by *ischemia*, but more so by *reperfusion following periods of ischemia* caused by AKI. In response to AKI/post-AKI reperfusion, the chain of cellular apoptosis is initiated in liver cells and also liver cells are stimulated to produce IL-6 and IL-10. Both of these cytokines are involved in liver regeneration and also in hepatocarcinoma development. Moreover, not only pre-renal AKI can imbalance cytokine release from kidney and create effects on liver cells. Bacterial infections, cancer, autoimmune diseases, all can be AKI developers and also play a role of *cytokines dysregulation* in the liver.

Another aspect of liver-kidney crosstalk is maintaining proper ***acid-base balance***. The liver and kidneys are responsible for eliminating the lactic acid (produced by anaerobic pulmonary cells**.** When renal clearance of lactic acid is impaired due to AKI, the kidney has a "chat - crosstalk" with the liver in order to maintain a normal ph.

A well-known, intricate and wide clinically investigated liver-kidney interplay is suggest by the onset of glomerulonephritis (GN) secondary to chronic liver infections (HBV, HCV infections). HCV infections are associated with various form of GN, including cryoglobulinemia induced GN which suggests that hepatorenal crosstalk goes beyond HRS. HCV's ability to attach and invade renal parenchyma via CD81 and SR-B1 receptors and HCV RNA in mesangial, tubular, and endothelial cells of glomerular and tubular capillaries is thought to start the pathogenesis. HCV-induced mixed cryoglobulins captured in the glomerular capillaries and mesangium contribute to GN.

Another recent example of a recognized and researched two-way crosstalk between the liver and kidney, in terms of illness, is the crosstalk between the accumulation of fatty droplets in the liver and their influence on CKD development and progression, as well as the potential influence of chronic renal damage on overloading the liver fatty droplets. Even though research into the pathopsychological mechanisms underlying this contentious interaction is ongoing, it appears that oxidative stress, insulin resistance, inflammatory pathways, gut microbiota, fructose metabolism, the RAS system, and possibly even some hepatokins are all involved in this process.

As mentioned in this thesis, T2DM patients are more likely to develop and worsen NAFLD. T2DM and NAFLD's controversial link is insulin resistance. Hepatic lipogenesis changes increase gluconeogenesis and insulin signals. Insulin resistance accelerates CKD by altering renal hemodynamics, salt excretion, and natriuretic peptide production via sympathetic nervous system activation .

In fatty liver disease, adipose tissue inflammation increases systemic insulin resistance and inflammatory pathways that may affect kidney function. Liver inflammation increases IL-1, IL-6, and TNF-α production. At this point in the disease, the liver is both victim (free fatty acids and chronic inflammation) and aggressor (amplifying inflammation by activating NF-kB pathway).The pathogenic mechanism by which fatty liver pro-inflammatory cytokines damage kidneys is studied.

The role of TNF-α, a pleiotropic cytokine, is to promote immune cell activation and recruitment and to trigger cell proliferation, differentiation, apoptosis, and necroptosis. It also mediates the inflammatory response. Two receptors, TNFR1 and TNFR2, mediate those effects. TNF-α and its receptors' function in renal damage is not fully understood. Experimental studies on animals indicate that administration of TNF-α to rat models exacerbate the severity of anti-glomerular basement membrane antibody-mediated nephritis and also other studies suggest that blocking TNF-α correlates with decreasing levels of proteinuria, lower inflammatory status, lower incidence of renal scarring and crescents, tubular cell death, lower caspase activity, and lower levels of various markers of renal fibrosis. High levels of circulating TNFRs were related to the incidence and progression of CKD in human trials and also that TNFRs impacts the CV outcome of CKD patients.

From IL-1 family the most important systemic proinflammatory effect is induced by IL-1α and IL-1ß. Fatty livers release IL1α. After monocyte enzymatic cleavage, IL-1ß initiates acute phase response in the liver via C-reactive protein. Circulating IL-1 directly affects kidney function is under investigation. NLRP3, IL-1α, and IL-1ß-deficient mice were protected from renal cell inflammation and necrosis in AKI rat models. Thus, there is little evidence that the NLRP3 inflammasome and IL-1 cause CKD. Systemic IL-1 release likely causes systemic endothelial dysfunction, which promotes leukocyte adhesion and kidney vascular leakage (albuminuria) and may accelerate CKD. Although the kidney clears IL-6, chronic inflammatory conditions like fatty liver disease, where proinflammatory cytokines are constantly released, can damage renal function. IL-6 suppresses adiponectin, which speeds up CKD progression and vascular disease and atherosclerosis.

Nonetheless, CKD in fatty liver patients could originate from pathophysiological pathways unconnected to inflammation of the liver. Hepatokines like adiponectin and the liver-secreted protein fetuin-A may have significant functions in regulating the crosstalk between adipose tissue, the kidney, and the liver, according to recent studies.Since diabetes patients were advised to replace sugar with low-glycemic index sugar substitutes like fructose, fructose metabolism has become an important area of study. Some processed foods use fructose-based corn syrup. Observational studies have linked corn syrup consumption to NAFLD and CKD. There is also some proof that gut microbiota plays an important role in the etiology of both liver fat overload and CKD. And also, when the degenerative axis (angiotensin II (Ang II), angiotensin converting enzyme (ACE), and angiotensin II type 1 receptor (AT1R)) is activated, the RAS system is suggested to be involved in this complex liver-kidney interaction .

SPECIAL PART

Our research seeks to show that the range of chronic liver disease that can contribute to the onset and progression of CKD is broader, involving more pathology, than chronic hepatitis viral infection or hepatorenal syndrome, such as metabolic liver diseases. We focused our research on proving the link between MAFLD and CKD, as well as assessing new, non-invasive methods for sustaining this aspect of the hepatorenal crosstalk.

The first study presented in this thesis addresses a much discussed and studied hepato-renal interaction between chronic hepatitis B and C viral infection and ESKD population.

Our first study aimed (1) to *reevaluate the prevalence of hepatitis virus infections in the HD population in Romania after 5 years of oral DAAs therapy* and (2) to *evaluate the influence of DAAs on the outcome of HD patients*. The study was conducted *retrospective observational* with the purpose of comparing two cohorts of ESKD patients who received HD therapy in ten HD centers in Romania in 2015 and 2019, respectively. The 2015 cohort consisted of 1401 patients, whereas the 2019 cohort included 1698 patients from the same HD centers as in 2015. We also tracked and evaluated the patients' survival rates from 2015 to 2019. The *clinical protocol of the study* required only ESKD patients who had completed more than 90 days of HD therapy to be enrolled in the study. Blood samples were collected. Comorbid conditions were also evaluated.For HD therapy high-flux, high-surface, single use polysulfone filters and B. Braun acidic bicarbonate hemodialysis concentrate was used. Surface sterilization was performed after each HD session, and "no touch" protocols were implemented for handling the catheters. Patients with HV infections have traditionally been treated in special wards and all staff had negative markers for HV infection. *Exclusion criteria* applied in the research were: patients with renal transplants; patients transferred to other HD facilities during the follow up; patients switched to other renal replacement therapies; patients treated for less than 3 months. *The follow-up* period was twelve months. *Statistical analysis* was performed using MedCalc v19.3 program.

The results of our research showed that in the 2015 cohort of 1,401 HD patient, the average prevalence of HBV infection was 4.7% (ranging between 2.4% and 5.6%), and the prevalence of HCV infection was 16.9% (ranging between 11% and 27.9%). Although the mean prevalence of HBV infection remained the same in the 2019 cohort at 4.8% (ranging between 3.6% and 5.4%), the absolute number of cases increased primarily due to new entries made prior to the 2019 cross-sectional analysis. The percentage of people who tested positive for anti-HCV antibodies fell to 10.5% after a nationwide program of DAA therapy was carried out for a period of 5 years (2015-2019). Despite the low percentage of HVC infected patients who accepted DAA therapy in 2015 (20.2%), after the required period of follow-up, all patients became negative for the virus; results demonstrated by 3 PCR determinations. It is very important to keep in mind that the DAA program was not intended solely for HD patients, but rather for the infected general population as well as other groups at a high risk of contracting the disease. As a result, it is reasonable to anticipate that there will be a reduction in the number of newly infected entries into HD centers.

Our data from the 2015 cohort demonstrated a significant decline in the prevalence of HBV infection in the HD patients from the previous study conducted in 2010, with a decrease from 9.5% to 4.7%. Moreover, the prevalence remained stable over the following five years, as indicated by the 2019 cohort findings that reported a prevalence of 4.8%.

Regarding mortality risk, in our study of 2015 cohort, conducted before the implementation of DAA therapy in Romania, we found that the presence of HCV antibodies (indicative of HCV infection) increased the risk of mortality and no significant effect on mortality risk was registered in relation with HBV infection.

Further analysis of the cohort showed that 44.1% of the patients (619 patients) survived after 5 years, with survivors accounting for 36.4% of the 2019 cohort. We also found that the 5-year mortality rate differed depending on the infection status of the patients. For those infected with HBV, the 5-year mortality rate was 45.4%. However, for HCV-infected patients who received DAA therapy, the rate was 20.8%, while for those who did not receive treatment it was 63.3%. For non-infected patients, the 5-year mortality rate was 55.7%.

Our analysis of the 2019 cohort revealed that mortality risk was significantly impacted by age (HR 1.02 , p=0.0007). When comparing the group of patients with HV infection who underwent a 5-year DAA therapy program from both the 2015 and 2019 cohorts, we observed that the 2019 group had lower levels of inflammation (CRP p<0.0001, Ferritin p=0.0001) but a higher prevalence of coronary artery disease (p<0.0001). The 2019 group also had a lower prevalence of peripheral artery disease (p=0.009), stroke (p <0.0001), and heart valve calcification (p=0.035). Furthermore, we found a significant difference in one-year mortality rates between the HV infected group before DAA therapy (2015 cohort) and after DAA therapy (2019 cohort) (12.2% vs 6.8%, p=0.017).

In conclusion, after 15 years of a nationwide infection prevention program for HV infections and 5 years of DAAs treatment in Romania, the prevalence of HBV did not change but HCV infections decreased significantly, however, it still remained high. It is noteworthy that HCV virus infection was no longer associated with mortality in 2019, which is a positive outcome of the national prevention and treatment efforts.

As previously stated in this thesis, considerable literature evidence links non-alcoholic fatty liver disease (NAFLD) with type 2 diabetes, and other metabolic illnesses. Given that nearly all people with CKD have atherogenic dyslipidemia in some form, changing the concept of NAFLD to metabolic dysfunction associated fatty liver disease (MAFLD) may widen the pathological hypothesis to also include chronic renal lesions and, possibly, adjust the epidemiological correlation between CKD and liver damage. In light of all of these changes, ***our next research focus was to examine the relationship between fatty liver overload which recently was defined as MAFLD and CKD*** in order to prove the metabolic involvement in hepatorenal crosstalk.

The research design was *prospective*, and follow-up period was one year (January 2018 to December 2018). We enrolled in our study 402 diabetic individuals. Every patient who was included in the study undergo imaging methods such as TE with CAP. This imaging methods were applied so that we could determine whether or not a patient had MAFLD. CKD was defined accordingly with KDIGO guidelines and GFR was calculated using CKD-

Epi formula. Exclusion criteria were represented by pregnancy, ascites, outliers, decompensated liver disease, cardiac pacemaker, malignancy, end-stage renal disease, heart failure, unreliable or invalid TE and CAP measurements, and elevated AST and ALT levels, liver cirrhosis. After appliance of exclusion criteria 335 individuals remained included in study. Blood and urine samples were obtained. To ensure that the diagnosis of CKD was correct we determined serum creatinine levels and calculated GFR at 1, 2 and 3 months from the beginning of the study. TE with Cap was performed using FibroScan® (Echosens, Paris, France). Staging fibrosis and steatosis was acquired by using the cut-off values of CAP and TE from a previously published multicentric study compared with biopsy. Statistical analysis was performed using MedCalc software (version 19.3.1) and Microsoft Excel 2019.

The participants in our study had a mean age of 60.84 ± 9.11 years and were all diagnosed with type 2 diabetes. Our sample size was three hundred thirty-five (N=335). The majority of the people in our cohort were male, and they had poor glycemic control (the median fasting glucose was 180.38 ±60.63, and the median glycosylated hemoglobin (HbA1c) was 8.53 ± 1.80). The prevalence of hypertension was also high (83.8%), which was in line with our expectations. In addition, our group had high levels of cholesterol (189.22 ± 64.76), and CKD was discovered in 60.8% of the patients we enrolled. Regarding the transient elastography with controlled attenuation parameter findings, in CKD patients we recorder higher mean liver stiffness measurements (LSM) values and higher mean-controlled attenuation parameter (CAP) values. Similar rates of fibrosis and steatosis were found in CKD patients. ROC curves were applied in order to determine if the TE with CAP might predict the existence of chronic kidney lesions by using the evaluation of liver stiffness or liver steatosis measurements. Based on the results of the ROC curve analysis, we found that the controlled attenuation parameter (CAP) is a more accurate predictor for the incidence of CKD than the transient elastography (TE) parameter. Additionally, the optimal CAP cut-off value had a sensitivity of 75% and a specificity of 45.1%, and it was 353 dB/m. We used univariate and multivariate logistic regression analysis to find factors involved in CKD prediction. In the univariate analysis, we found that older age ($p<0.0001$), male gender ($p<0.0001$), higher levels of HBA1c ($p = 0.002$), higher levels of fasting glucose ($p = 0.04$), higher CAP values ($p = 0.03$), use of angiotensin-converting enzyme (ACE) inhibitors ($p = 0.04$), and higher serum creatinine levels (p0.001) were all significantly associated with CKD. Our hypothesis was supported by the stepwise multiple logistic regression analysis also. After adjusting for gender, fasting glucose, body mass index, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, CAP value, age, and hemoglobin A1c, controlled attenuation parameter (CAP) remained associated with CKD presence in multivariate logistic regression analysis and not fibrosis or LSM values. To exclude bias factors like hypertension, dyslipidemia, fibrate treatment, and diabetes duration, we used regression models with CAP values to predict CKD. Higher CAP values independently predicted CKD in all models.

In conclusion, our study demonstrates that in patients with established MAFLD and multiple metabolic risk factors for CKD, the amount of liver fatty droplets evaluated with controlled attenuation parameter (CAP) is a better predictor of CKD than liver stiffness measurements (LSM) and fibrosis. This suggests that liver fat overload may be an important

factor contributing to the development of CKD in these patients. However, the study acknowledges that further research with a larger number of patients is needed to confirm these results. In addition, the study raises important questions for future research regarding the liver-kidney crosstalk and how CKD may influence MAFLD outcomes. While CKD is typically considered a one-way pathological process, the study highlights the need to investigate how CKD may impact the progression of MAFLD. Furthermore, the study also raises the question of whether MAFLD may influence the progression of CKD to ESKD. This is an important area for further investigation as it could provide insight into the complex interplay between MAFLD and CKD, and could have important implications for the management and treatment of both conditions.

The last study included in this thesis, goes further and evaluates the utility of new non-invasive ultrasound tools as contrast enhanced ultrasonography with arriving time parameter (CEUS-PAT) and his potential for diagnosing chronic kidney disease (CKD). Also, if we suppose that cytokines and the microbubbles of UCA used for CEUS investigation have almost the same behavior in bloodstream, our research may show us the route of cytokines travelling through bloodstream based on the route made by Sonovue microbubbles used for CEUS-PAT.

The third study included in this thesis was conceived in two parts . *The primary purpose* of the study was to determine if in cirrhotic patients, liver fibrosis can be reliably assessed using contrast-enhanced ultrasonography with arrival time parametric imaging. *The second part* of the study explored the relationship between the presence of chronic kidney disease and its association with impaired measurements of contrast-enhanced ultrasonography with arrival time parametric imaging. *The design of the study* was *case-control* study which included 64 patients (27 healthy volunteers and 37 cirrhotic patients). Patients were enrolled in a period of twelve months, from January 2018 until December 2018. VCTE investigation was the used reference test.

The first part of the study used LOGIQ E9 (GE Healthcare, Chalfont St.Giles-UK) probe C1-6 for ultrasonography. Each subject underwent CEUS with SonoVue (Bracco SpA, Milan, Italy) as the contrast medium (1/2 vial). After dissolving the substance in 5 mL saline, 2.5 mL of dissolved contrast agent was injected into the cubital vein, followed by 5 mL of regular saline. Fasting lasted at least 12 hours. The liver and right kidney CEUS data were saved on a hard drive within 30 seconds of contrast agent injection through the cubital vein. Following ultrasound acquisition, parametric imaging was performed using the ultrasound system's dedicated image analysis software. The specialized software provided arrival time parametric imaging (AtPI) values of the contrast agent, named - the CEUS-PAT method. When the parametric image button is pressed, the system will automatically generate a color map on the CEUS image. Using the arrival time of the contrast into the kidney and the arrival time of the contrast into the liver, a ratio of the arrival parametric time was calculated. VCTE by using FibroScan® device (EchoSens, Paris, France) was also performed and the data was blinded for the CEUS investigator. Fibrosis scores such as APRI and FIB -4 score were also calculated for each patient. Blood samples were obtained. The inclusion criteria for patients with cirrhosis were: age>18 years, positive diagnostic of cirrhosis based on clinical, paraclinical, endoscopic, ultrasonographic and elastography data; for healthy volunteers –

no clinical, biological and ultrasound signs of any other disease. Exclusion criteria were represented by : ascites, portal hypertension, pregnancy, cardiac pacemakers, malignancy, ESKD, heart failure, unreliable or invalid VCTE and CAP measurements, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) more than five times the upper limit of normal values. In the second part of the study chronic kidney disease was assessed.

CKD was defined according to KDIGO guidelines by estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2. The eGFR was estimated using the CKD-Epi formula. The statistical analysis was performed using IBM SPSS Statistics v. 20.0.0(New York, NY, USA) and MedCalc software (v. 19.3.1, Ostend, Belgium). A boot resampling method was also applied.

Our study CEUS-PAT results confirm that when comparing liver cirrhosis group of patients with healthy volunteers, in the liver cirrhosis group higher mean values of controlled attenuation parameter (CAP) were found (269.86 ± 63.46 in liver cirrhosis patients versus 223.74 ± 41.25 for healthy volunteers, p =0.0016). Also, VCTE mean values registered in cirrhotic patients were more elevated than in healthy volunteers 20.21±14.97 versus 5.87± 2.87, respectively, with a p <0.0001. CEUS- PAT evaluation respected the same trend, registering a mean value of 0.83±0.09 in the liver cirrhosis group versus 5.87±2.87 in healthy volunteers with p<0.0001. *The optimal cut-off values* of CEUS -PAT for detecting liver fibrosis were also evaluated. According to Youden's index, *the optimal ratio AtPi was > 0.7* with a sensitivity of 89.19 % and a specificity of 100% (AUC 0.98, p<0.001). *The univariate and multivariate analysis* was performed in order to find factors associated with high CEUS-PAT measurements in patients with cirrhosis. In the univariate model hemoglobin (p = 0.04), sodium (p = 0.003), AST (p = 0.01), ALT (p = 0.02), albumin (p < 0.0001), and severe steatosis at CAP (p = 0.03) were associated with CEUS-PAT values. In the multivariate analysis only albumin levels remained associated with CEUS-PAT measurements. Furthermore, a boot resampling analysis was performed and the results were similar to the asymptotic methods.

Regarding the presence of CKD, we found that from 37 subject with an established diagnostic of liver cirrhosis – 20.2% (N=13) had also chronic kidney disease (eGRF < 60ml/min/1.73m) and none of them accomplished chronic kidney disease criteria. Furthermore, the liver cirrhosis patients were split into two groups, with and without CKD, for assessing the AtPi in each group (non-CKD and CKD). We found a significant difference between the ratio of the arrival time of the contrast agent (AtPi) into the kidney and liver, 0.85 ± 0.09 for CKD patients vs 0.65 ± 0.19, non-CKD patients, p=0.0005.

We also explored some models to appreciate what are the factors associated with kidney disease in patients with liver cirrhosis and CKD and we found that liver steatosis (p<0.0001) , age over 60 years (p=0.01) and low albumin levels (p<0.0001) were significantly associated with CKD presence.

As a conclusion of our research when it comes to the liver, contras enhanced ultrasonography with arriving time parameter (CEUS-PAT) is an efficient method for diagnosing liver cirrhosis that can be utilized even in situations in which the use of other diagnostic techniques is not a viable option. When applied to the process of diagnosing cirrhosis of the liver, it demonstrates outstanding performance. Nevertheless, additional

research needs to be done on the various stages of liver fibrosis, and the ultrasound diagnostic algorithm for focal liver lesions needs to be improved. Both of these issues should be addressed in the near future. Specifically, the algorithm needs to be improved so that it can confirm or deny the presence of hepatocarcinoma. We can also state that there is a two-way relationship between the kidney and contrast enhanced ultrasonography with arriving time parameter in relation to the kidney. One is exemplified by the fact that the kidney arrival time of contrast agents is a useful tool for understanding liver disease, and the other is exemplified by the fact that CEUS-PAT appears to provide useful information regarding renal microcirculation changes that have occurred in both AKI and CKD in addition to renal masses or renal ischemia. Additionally, if we suppose that the proinflammatory cytokines follow the same pathways as the microbubbles used in CEUS research, we may understand why the liver's chronic disease influences the onset or progression of other chronic organ diseases (kidney) and reversely. However, these are only hypotheses, and more research is required to identify a pattern of this complex two-way relationship.

FINAL CONCLUSIONS

Non-inflammatory liver diseases like fatty liver disease can lead to kidney failure, adding to the known risk factors for kidney disease like chronic hepatitis B and C virus infections.

In Romania, efforts to reduce the burden of chronic viral hepatitis have decreased the prevalence of HCV infections among hemodialysis patients, but the prevalence of HBV infection remains high and associated with mortality risk in HD patients.

The relationship between chronic liver disease and CKD is broader, with MAFLD being a better predictor of CKD than liver fibrosis in patients with established MAFLD and multiple metabolic risk factors. Proinflammatory cytokines may play a significant role in this relationship.

CEUS-PAT is an efficient method for diagnosing liver cirrhosis and has potential for diagnosing other chronic organ diseases like CKD.

However, more research is needed to validate these hypotheses and identify a pattern of the complex two-way relationship between the liver and kidney.