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Clinical and pathophysiological characterization of patients with acutely decompensated cirrhosis and acute-on-chronic liver failure

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"Per natura vogliamo sapere di più.

Qui, sul bordo di quello che sappiamo,

a contatto con l'oceano di quanto non sappiamo,

brillano il mistero del mondo e la sua bellezza

e ci lasciano senza fiato."

(Carlo Rovelli)

TABLE OF CONTENTS

ABSTRACT

In the last decade, our understanding of the pathophysiological mechanisms underlying decompensated cirrhosis has greatly increased. The classical vision of liver cirrhosis as a disease with mainly hemodynamic alterations has been integrated in a more comprehensive hypothesis that attributed to systemic inflammation a key role in the pathophysiological cascade. At the same time, due to the pivotal CANONIC study, acute-on-chronic liver failure (ACLF) was defined as a distinct syndrome with specific clinical and pathophysiological features, thus contributing to a further understanding of acute decompensating events.

During the three years PhD Program, our research activities aimed to provide some personal contribution in characterizing patients with acutely decompensated cirrhosis and ACLF from a clinical and a pathophysiological perspective.

As a first project, we addressed the clinical issue of predicting in-hospital development of ACLF in patients hospitalized for acute decompensation of cirrhosis. We analyzed data from 410 patients with cirrhosis, non-electively admitted to hospital, and consecutively enrolled in a prospective observational study. We found that MELD score, leukocyte count and hemoglobin levels at admission could independently and reliably predict the development of nosocomial ACLF. Moreover, we defined a threshold of risk for each parameter and showed that the global risk of ACLF increased with the number of concomitant risk factors.

As a second contribution, during a research period abroad with a Marco Polo scholarship, I had the opportunity to deepen the molecular mechanisms underlying acutely decompensated cirrhosis. We performed a reassessment of the whole metabolomic dataset obtained from 831 patients enrolled in the CANONIC study, focusing on amino acids, with the aim to uncover potential alterations in amino acids metabolic pathways. The analysis provided a further tile in the complex mosaic of ACLF pathophysiology, showing that in patients with ACLF amino acids may be mobilized from skeletal muscles or derived from Krebs cycle intermediates to fuel anabolic programs, including protein and nucleotide synthesis, engaged by the intense innate immune responses. Ketogenic amino acids

were extensively catabolized, probably to produce energy substrates in peripheral organs, but the insufficient effect led to organ failures.

As a third perspective, we performed a GWAS on 270 patients with acute decompensation and ACLF included in the first clinical study. DNA samples from 319 patients were available, but 49 samples were excluded due to various quality issues. We categorized patients in 4 groups, according to their clinical presentation during hospitalization, and their clinical course during follow up. Therefore, groups 1 (n=76) and 2 (n=80) included patients that developed ACLF or bacterial infection(s) during hospital stay, differentiated by their survival status at 1 year (group 1, dead; group 2, alive). Groups 3 (n=38) and 4 (n=76) included patients who remained free from ACLF or infection(s), differentiated by their survival status at 1 year (group 3, dead; group 4, alive). We then performed two comparisons: group 1 vs group 4 (i.e., the *most severe* vs the *mildest* clinical courses), and group 1 vs group 2 (i.e., patients with different 1-year outcomes from a common clinical presentation with ACLF or bacterial infection). Three SNPs (rs9354118 on chromosome 6q16.1; rs1146878 on chromosome 13q22.2; rs6479397 on chromosome 9q22.31) were significantly associated with the selected phenotypes, but all of them were located in non-codifying DNA regions. However, their potential role as candidate Cis-Regulatory Elements (cCREs) opened interesting hypotheses on effects on the expression of neighboring genes. Indeed, four of them (FUT9 and UFL1 for SNP rs9354118, and LMO7 and ACOD1 for rs1146878) are involved in the modulation of immune system activation and systemic inflammation, thus uncovering a potential pathophysiological interest for patients with acutely decompensated cirrhosis and ACLF.

The results of the GWAS did not confirm previous findings reported in literature (although scarce and partially not comparable) and presented some methodological limitations. However, it provided the basis for further research in this still open issue.

1. INTRODUCTION

The natural history of liver cirrhosis is classically divided in two different stages: a generally asymptomatic and slowly progressive compensated phase, and a more advanced decompensated phase characterized by a tumultuous clinical course. The transition between these two different stages of the disease occurs at a rate of about 5-8% per year and carries a dramatic worsening of life expectancy, as patient median survival drops from more than 12 years for compensated cirrhosis to about 2-4 years for decompensated patients [1,2]. Moreover, the decompensated stage of the disease represents a heavy burden of social and healthcare costs, since patients enter a clinical course punctuated by further complications and frequent hospitalizations, thus worsening their quality of life and significantly affecting care-givers' commitment. The occurrence of ascites, portal hypertensive gastrointestinal bleeding, hepatic encephalopathy, and deep jaundice -alone or in combination-, represents the classical hallmark of this transition, along with a high susceptibility to bacterial infections [1,3]. At this stage, cirrhosis becomes a systemic disease with multi-organ dysfunction and failure.

In the last decade, a growing amount of clinical and experimental evidence heavily enriched our understanding of pathophysiological mechanisms underlying decompensation in cirrhosis. The classical pathophysiological paradigm has been integrated with more recent evidence. Moreover, even the strict distinction between compensated and decompensated stage has been questioned, and a more comprehensive vision has been proposed. These steps forward, however, more than representing a point of arrival, widened our vision to a new landscape that deserves to be furtherly explored and uncovered [4–6]. In this perspective, the institution of large international research networks and consortia, and the integration of multiple "omics" approaches and bioinformatic techniques, represent powerful tools in researchers' hands for years to come.

During the three years PhD Program, our research aimed to provide a personal contribution to the characterization of the clinical course of advanced cirrhosis, along with a deeper understanding of the pathophysiological mechanisms underlying its acute and severe clinical manifestations.

1.1. Pathophysiology of decompensated cirrhosis: an evolving paradigm

The first theories addressing the pathophysiology of decompensation in patients with cirrhosis date back to the end of the 19th century. At that time, three researchers in three different European cities explored for the first time the mechanisms underlying the development of ascites, hepatic encephalopathy, and gastrointestinal hemorrhage.

In London, Ernest Starling proposed that ascites could develop due to the combination of an increased hydrostatic and a low oncotic pressure at the sinusoidal capillaries [7]. The subsequent net fluid flow towards the interstitium leads to an excessive lymph production, that progressively overwhelms the thoracic lymphatic drainage, and reaches splanchnic cavity through the glissonian membrane, thus causing ascites formation. The reduction in circulating blood volume has been subsequently proposed as the cause of renal sodium and water retention, according to the so called "underfilling theory" [8]. In the same period, in Saint Petersburg, Pavlov and Colleagues investigated the pathogenesis of hepatic encephalopathy, through fundamental studies on animal models. They obtained the clinical manifestations of hepatic encephalopathy in dogs undergoing portocaval anastomosis (the so called "Eck's fistula"). The simultaneous measurement of a marked increase in the urinary excretion of ammonium salts allowed them to reproduce the symptoms through the oral administration of this molecule [9–11]. Finally, in Paris, Augustine Gilbert first theorized the concept of hypertension in the portal system as a consequence of liver cirrhosis in his book "Les Fonctions Hépatiques". The development of hypertension in the portal system could cause the enlargement of natural collaterals between the portal and the systemic venous circulation, with subsequent occurrence of esophageal and gastric varices [12].

During the 20th century, and especially after World War II, new experimental evidence brought novel elements in the discussion, thus leading to a reorganization and revision of the previous concepts. One of the most important is the discovery of an imbalanced activity of vasodilators (i.e., nitric oxide [NO], carbon monoxide [CO], endocannabinoids, hydrogen sulphide) and vasoconstrictors (i.e., angiotensin II and endothelin), that represents the *functional* component of portal hypertension [13–15]. Indeed, this condition is not only the result of a *mechanical*

distortion of the hepatic histological architecture due to liver fibrosis and regenerative nodules. The occurrence and progression of portal hypertension is rather the combination of an increased intrahepatic vascular resistance -initiated by the fibrotic rehash of liver parenchyma and sustained by a prevalent vasoconstrictive effect- and an excessive inflow to the portal venous system from the splanchnic circulation, because of a dysregulated NO release and activity [16,17].

1.1.1. The peripheral arterial vasodilation hypothesis

A comprehensive reformulation of ascites pathophysiology has been proposed in 1988 by a group of international experts that systematically collected the new evidence in the so called "Peripheral arterial vasodilation hypothesis" (PAVH), that represented for almost three decades the most advanced theory on decompensated cirrhosis [18]. According to this hypothesis, the pivotal event of the pathophysiological cascade is the development of effective hypovolemia, whose connection with portal hypertension is represented by a dysregulated production of endogenous vasodilating substances (NO, CO, endocannabinoids) that mainly exert their action on the splanchnic arteriolar bed. This dysregulated splanchnic vasodilation seizes blood volume and favors a condition of relative (or *effective*) hypovolemia. The activation of neuro-humoral systems, such as the renin-angiotensin-aldosterone (RAA) axis, the sympathetic nervous system (SNS), and arginine-vasopressin (AVP), in parallel with an increase in cardiac output, are all homeostatic responses put in place to promote vasoconstriction and renal retention of sodium and water. These compensatory mechanisms induce a state of hyperdynamic circulation, with the aim to maintain the perfusion of peripheral organs. As disease progresses, however, these mechanisms can lead over time to a microvascular dysfunction and the exhaustion of left ventricular function (also favored by the development of cirrhotic cardiomyopathy), with a secondary impairment of cardiac output and a further decrease of effective volemia, ultimately leading to peripheral hypoperfusion and contributing to multi-organ failure [18]. This pathophysiological interpretation, however, does not fully explain all the clinical manifestations of decompensated cirrhosis, with the subsequent need to integrate it with recent fundamental achievements.

1.1.2. Systemic inflammation: a fundamental pathophysiological background

Patients with decompensated cirrhosis generally express patterns of immune activation, mainly represented by an increased white blood cell (WBC) count and C-reactive protein (CRP) levels. This is particularly true for acutely decompensated patients, since less than 1% of them do not present any evidence of systemic inflammation. Moreover, blood levels of circulating inflammatory cytokines (among which TNFa, IL-6, IL-8, IL-10 and IL-1ra are the most important) increase in parallel with disease stage and the severity of acute decompensation [19–21]. On this basis, systemic inflammation has been proposed as a key mechanism in the progression of cirrhosis to the decompensated stages until multi-organ impairment and failure [4–6]. Portal hypertension and chronic liver damage (with hepatocytes necrosis) are the two major drivers of immune system activation, through different but complementary mediators.

On one hand, portal hypertension impairs splanchnic microcirculation leading to venous congestion and increased permeability of the intestinal mucosa. This condition, combined with the intestinal bacterial overgrowth and changes of the gut microbiome, allows the translocation of bacterial products, also defined pathogen-associated molecular patterns (PAMPs) that are recognized by innate myeloid cells (i.e., monocytes and neutrophils) and other cells of the innate immune system through dedicated pattern-recognition receptors (PRRs, such as Toll-like receptors [TLRs] and NOD-like receptors [NLRs]). PRR engagement stimulate intracellular signaling cascades, ultimately leading to the transcription and synthesis of inflammatory mediators [22,23]. Common PAMPs are LPS, flagellin and bacterial or viral DNA/RNA. The engagement of TLR4 by LPS, derived from the cell wall of Gram-negative bacteria, is a classical paradigm of these mechanisms [24]. On the other hand, damaged or dying host cells (mainly hepatocytes, due to chronic liver damage) release circulating damage-associated molecular patterns (DAMPs), that bind and activate specific PRRs, also defined inflammasomes [25]. DAMPs can derive from various cellular compartments: they consist of nuclear (high mobility group box-1 [HMGB1], histones), cytosolic (ATP, members of the S100 calcium-binding protein family [S100A8, S100A9, and S100A12], cholesterol, and urate crystals) or mitochondrial components (fragments of mitochondrial DNA and formyl peptides).

Different forms of liver injury (e.g. alcoholic hepatitis, ischemic liver injury, or HBV-related submassive hepatic necrosis) are well-known causes of DAMPs release, with different molecular profiles and receptors [26–28].

1.1.3. Outcomes of systemic inflammation

The binding and recognition of PAMPs and DAMPs by the innate immune cells induce the release of pro-inflammatory mediators that exert multifaceted effects. From a hemodynamic perspective, they can activate inducible nitric oxide synthase (iNOs) in splanchnic arteriolar walls, thus increasing splanchnic vasodilation, worsening effective hypovolemia, and further triggering overactivation of endogenous neurohumoral vasoconstrictor systems. Neurohumoral mediators particularly affect the renal circulation, causing kidney hypoperfusion, decreased glomerular filtration rate (GFR) and acute kidney injury (AKI) [4]. Beyond hemodynamic effects, there is evidence of a phenomenon defined immunopathology, or the immune-mediated tissue damage from activated immune cells. For example, TNF-α and NF-κB-dependent signaling pathways may play a role in impaired left ventricular contractility, NO-mediated pulmonary dysfunction with macrophage accumulation, and hepatocyte apoptosis [29–31]. Moreover, capillary leukocyte infiltration, vascular microthrombosis, and cell apoptosis may induce a sepsis-like AKI in absence of renal hypoperfusion [32]. As a vicious cycle, immunopathology can sustain and exacerbate systemic inflammation through the release of cellular products acting as DAMPs, thus providing the mechanistic link between systemic inflammation, cell injury and organ failure [23]. The current paradigm on decompensation in cirrhosis, integrating the peripheral arterial vasodilation hypothesis and the systemic inflammation hypothesis, is represented in Figure 1.

Finally, recent metabolomic and lipidomic analyses of a large cohort of patients with acutely decompensated cirrhosis, showed profound changes in cellular energetic metabolism, that still deserve further understanding [33,34]. On one hand, the overwhelming (and energetically expensive) activation of immune cells requires a metabolic trade-off in the host and causes an energetic shift from peripheral organs to immune cells. Consequently, parenchymal cells are forced

to alter their metabolic activity by enhancing proteolysis and lipolysis. In addition, systemic inflammation can affect mitochondrial functions and impair both oxidative phosphorylation and fatty acid beta-oxidation, thus precluding appropriate energy production in peripheral organs, and ultimately leading to organ failure [35–37].

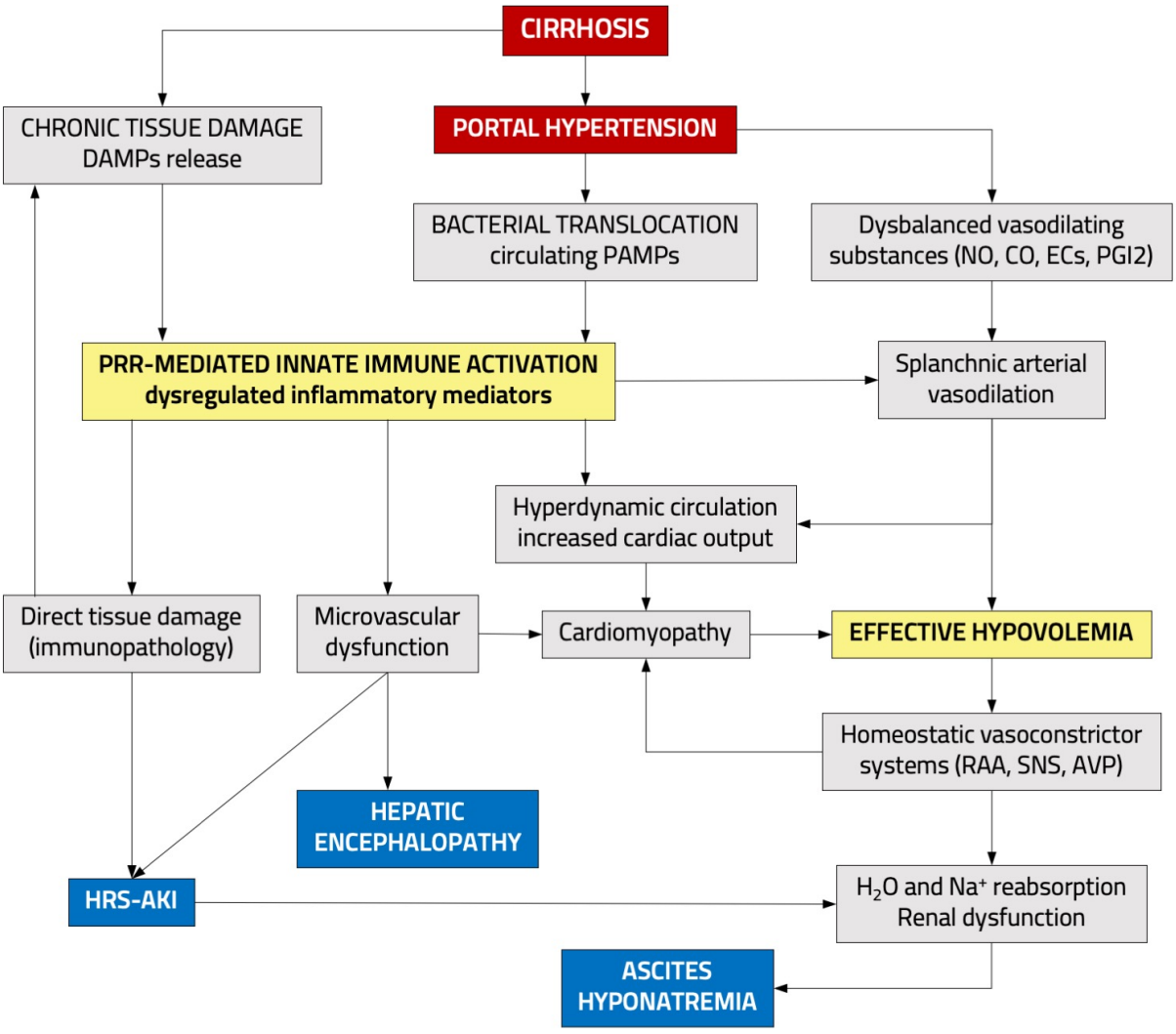


Figure 1. Comprehensive pathophysiological vision integrating the "Systemic inflammation hypothesis" (SIH) with the classical "Peripheral arterial vasodilation hypothesis" (PAVH), at the best of current knowledge. Effective hypovolemia was the central event in the PAVH, whereas the dysregulated overactivation of immune system plays a pivotal role in the SIH. See text for further details. DAMPs: damage-associated molecular patterns; PAMPs: pathogen-associated molecular patterns; NO: nitric oxide; CO: carbon oxide; ECs: endocannabinoids; PGI2: prostacyclin; PRR: pattern recognition receptor; RAA: renin-angiotensin-aldosterone; SNS: sympathetic nervous system; AVP: arginine vasopressin; HRS-AKI: hepatorenal syndrome acute kidney injury.

1.2. Clinical course of decompensated cirrhosis: acute and non-acute decompensation

As for any chronic disease, defining the evolutionary stages and their prognosis is of primary importance to guide clinical management and therapeutic strategies. We already remarked the distinction between the slowly progressive and generally asymptomatic compensated stage of cirrhosis, in contrast with the turbulent decompensated phase. Both the prognostic significance and the therapeutic management of these disease stages compose two completely different clinical scenarios. The watershed between them is classically marked by the development of complications (mainly ascites, gastrointestinal bleeding, and hepatic encephalopathy) [1,2]. However, even if the general concept of "decompensation" is universally accepted as the most important stratification variable, both for prognosis and therapeutic approaches, a unanimous consensus on its precise definition is still far from being reached [1].

At the same time, it has been recently claimed that the dual classification of cirrhosis in compensated and decompensated could be too simplistic and assimilate different prognostic subgroups. Indeed, considering the two most frequent decompensating event (ascites and variceal bleeding) and their different combination, up to five different prognostic stages of disease have been identified and proposed [38]. Ascites represents the first decompensating event in more than 70% of patients with cirrhosis, a half of them presenting ascites alone, and the remainder in combination with other complications. Its prognostic significance is worse than variceal bleeding alone, but their combination is associated with the worst outcomes. Therefore, the type of decompensation (or the combination of different decompensating event) can affect patient outcomes in different ways and with different prognostic impact [38].

A step in this direction is represented by the proposal to classify decompensated patients with very advanced disease in a sort of further (or "late") decompensated stage. This group should include patients with various combination of bacterial infection, refractory ascites, hepatorenal syndrome, or recurrent hepatic encephalopathy. The 1-year mortality rate of such severely ill patients ranges between 60 and 80% [39–41]. Indeed, from a pathophysiological perspective, these conditions could identify a subgroup of patients with a more intense systemic inflammatory state,

which can trigger or amplify extra-hepatic organ failure, with circulatory and renal impairment as major clinical features.

Further information on this issue derives from the CANONIC and PREDICT studies, two large multicenter European studies that aimed to characterize acutely decompensated cirrhosis from different perspectives [21,42]. The CANONIC cohort showed that patients without previous history of decompensation (i.e., at the first acute decompensating event) showed more severe and intense clinical manifestations, as defined by the number of organ failures, laboratory markers of systemic inflammation, and overall mortality. Moreover, the PREDICT study revealed the importance of concomitant precipitating factors in determining clinical phenotypes and outcomes of acutely decompensated patients. Indeed, the number of precipitating factors rather than their nature was associated with patient outcome[43]. Interestingly, both in CANONIC and PREDICT cohorts, more than half of enrolled patients did not present any identifiable precipitating event [21,43].

As a further issue, the current concept and definition of decompensation in cirrhosis are mainly related to acute decompensating event, and do not apply to patients presenting slowly progressive decompensation. This is the case, for example, of many patients progressively accumulating mild/moderate ascites, and gradually developing jaundice, or mild grade hepatic encephalopathy [44,45].

All this considered, a step toward a better definition of decompensation in cirrhosis is represented by the proposal of two distinct modalities of transition from the compensated to the decompensated stage [45]. Indeed, the acute or non-acute onset of decompensation can stratify subgroups of patients according to their clinical phenotypes and prognosis. Acute decompensation could be defined as any first or recurrent moderate/tense ascites within 2 weeks, first or recurrent acute hepatic encephalopathy in patients with previous normal consciousness, acute gastrointestinal bleeding, and any type of acute bacterial infection. Patients generally require hospitalization for an appropriate management, and often present concomitant extra-hepatic organ impairment. Non-acute decompensation includes slow ascites formation, mild/moderate hepatic encephalopathy, or progressive jaundice. In this case, patients are generally manageable in an

outpatient setting, even when presenting with two or more decompensating events. These two conditions, finally, have also different places in the natural history of cirrhosis, since non-acute decompensation mostly characterizes the first decompensating event, while acute decompensation mostly represents further decompensation.

1.3. Acute-on-chronic liver failure

Focusing our research on acutely decompensated patients with cirrhosis, acute-on-chronic liver failure (ACLF) deserves a specific interest. The term has been first proposed more than 25 years ago to describe a condition of chronic liver disease with a superimposed acute deterioration of liver function [46]. The general concept is universally accepted, and it has been adopted to identify an abrupt and life-threatening worsening of clinical conditions in patients with cirrhosis or chronic liver disease. In the last decade, all the major international scientific societies proposed different definitions of ACLF, but a universal consensus on its diagnostic criteria is still far to be reached [21,47–49]. The most debated issues concern the type of precipitating event or insult (specifically hepatic, extrahepatic or both), the stage of the underlying liver disease (cirrhosis or chronic hepatitis) and the presence of concomitant extra-hepatic organ failures.

The first definition of ACLF was provided by the expert consensus of the Asian Pacific Association for the Study of the Liver (APASL) in 2009, and subsequently updated by the APASL ACLF Research Consortium (AARC) in 2014 and 2019. This definition applies to patients with chronic liver disease or compensated cirrhosis, at the first episode of acute liver function deterioration due to a specific hepatic insult (expressed by jaundice and coagulopathy), and complicated within four weeks by ascites or hepatic encephalopathy [47]. Patients with extrahepatic precipitants, or those with extra-hepatic organ failures are excluded of the definition. The classical paradigm of the Asian phenotype of ACLF is represented by HBV flare in patients with chronic hepatitis.

In 2013, the European Association for the Study of the Liver - Chronic Liver Failure (EASL-CLIF) Consortium proposed a definition based on the results of the CANONIC Study, a large observational prospective cohort of 1343 patients with cirrhosis (both compensated or already

decompensated), that were non-electively hospitalized for an acute decompensating event [21]. The EASL-CLIF definition is based on the combination of an acute decompensation (i.e., ascites, hepatic encephalopathy, gastrointestinal bleeding, acute jaundice, or bacterial infections), single or multiple organ failures (both hepatic and extra-hepatic) and a high short-term mortality (>15% at 28 days). The EASL-CLIF diagnostic criteria have been also validated in multiple external cohorts of patients, including Asian, South American, and North American cohorts [50–55].

The definition by the North American Consortium for the Study of End-stage Liver Disease (NACSELD), is based on observational data from 507 patients with acutely decompensated cirrhosis hospitalized for infection. Based on standard definitions of organ failures (i.e., shock, need for mechanical ventilation, need for renal replacement therapy and West Haven grade III or IV of hepatic encephalopathy) the North American criteria defined ACLF by the presence of two or more concomitant extra-hepatic organ failures. The definition was also validated on a large cohort of hospitalized patients precipitated or not by infection [48,56].

The above-mentioned differences in definition, diagnostic criteria and reference population also give rise to different clinical phenotypes of patients with ACLF [57]. As regards our research program, we referred to ACLF using the EASL-CLIF definition and diagnostic criteria.

1.3.1. EASL-CLIF diagnostic criteria for ACLF and scoring systems

The EASL-CLIF criteria for ACLF diagnosis apply to patients with cirrhosis at any stage of the disease (i.e., both compensated or with previous decompensations), which undergo an acute decompensating event, triggered by intra- or extrahepatic insult(s), and complicated by single or multiple organ failures. The diagnosis of organ failures was based on a modified Sequential Organ Failure Assessment (SOFA) scale (called CLIF-SOFA scale), subsequently simplified in the CLIF Consortium Organ Failure (CLIF-C OF) score, that consider the function of the six major organ systems, including liver, kidney, coagulation, brain, circulation, and respiration [58,59].

Based on the observational data provided by the CANONIC Study, ACLF criteria aimed to include patients with a high risk of short-term death (that was pre-specified as higher of 15% at 28

days). Therefore, ACLF diagnosis included patients with single kidney failure; those with single "non-kidney" organ failure if associated with kidney of brain dysfunction; and those with two or more organ failures. Accordingly, four groups of patients with acutely decompensated cirrhosis have been defined: a group of patients without ACLF and three groups of patients with increasing severity of ACLF (grade 1, grade 2 and grade 3) based on the type and number of organ failures [21]. Figure 2 reports the CLIF-C OF scoring system and the detailed criteria for ACLF grade definition.

Besides the CLIF-SOFA and the CLIF-C OF, the CLIF-C ACLF score was developed and validated to predict mortality among patients with ACLF. Based on the CLIF-C OF score, it included the two best independent predictors of mortality in the CANONIC cohort: age and WBC count [59]. The CLIF-C ACLF score performed better than the Model for End-Stage Liver Disease (MELD), MELD-Na, Child-Pugh-Turcotte and CLIF-C OF scores, in predicting patient mortality, both in the CANONIC population and in an external validation cohort. It also showed a better performance compared to the usual intensive care unit prognostic scores (including the Sequential Organ Failure Assessment [SOFA] and the Acute Physiology and Chronic Health Disease Classification System [APACHE] II scores). Moreover, the kinetics of the CLIF-C ACLF score during ICU stay reliably predicted patient outcome [59,60].

Organ/system	Variable	1 point	2 points	3 points
Liver	Bilirubin (mg/dl)	<6.0	≥6.0 to <12.0	≥12
Kidney	Creatinine (mg/dl)	<1.5 >1.5 to <2.0	≥2.0 to <3.5	≥3.5 or use of RRT
Cerebral	West-Haven HE grade	No HE	Grade I - II	Grade III - IV
Coagulation	INR	<2.0	≥2.0 to <2.5	≥2.5
Circulation	MAP (mmHg)	≥70	<70	Use of vasopressors
Lungs	PaO_2/FiO_2 $SpO2/FiO_2$	>300 >357	>200 to ≤300 >214 to ≤357	≤200 ≤214

Grade of ACLF	28-day mortality	90-day mortality
Grade 1 - type a: patients with single kidney failure Grade 1 - type b: patients with one "non-kidney" organ failure associated with serum creatinine ranging from 1.5 to 2.0 mg/dL and/or stage I-II hepatic encephalopathy	22.1 %	40.7 %
Grade 2: patients with two organ failures	32.0 %	52.3 %
Grade 3: patients with three or more organ failures	76.7 %	79.1 %

Figure 2. **Left panel**: Chronic liver failure-Consortium Organ Failure (CLIF-C OF) score [59]. The red and yellow boxes indicate the thresholds for organ system failure and organ dysfunction, respectively. RRT: renal replacement therapy; HE: hepatic encephalopathy; INR: international normalized ratio; MAP: mean arterial pressure; PaO2: partial pressure of arterial oxygen; FiO2: fraction of inspired oxygen; SpO2: oxygen saturation as measured by pulse oximetry. **Right panel**: ACLF grades and relative diagnostic criteria according to the EASL-CLIF definition and the corresponding 28- and 90-day mortality, as reported from the CANONIC cohort [21].

1.3.2. Clinical phenotype of ACLF according to the EASL-CLIF definition

Alcohol, chronic hepatitis C, and a combination of both were the most frequent cause of cirrhosis in the CANONIC cohort. The major triggers for acute decompensation were both hepatic (alcohol-induced liver injury) and extrahepatic (bacterial infections or gastrointestinal hemorrhage) events, although in up to 40% of patients no precipitating factors were identified. The kidney and the liver were the most prevalent failing organs (56% and 44% of ACLF patients, respectively), followed by coagulation (28%) and brain (24%), while cardiovascular and respiratory failures were less frequent (17% and 9% respectively). The 28-day transplant-free mortality was 32.8% in patients with ACLF compared to 1.9% in patients without ACLF. Mortality increased in parallel with ACLF grade (22%, 32% and 77% for grade 1, grade 2 and grade 3 ACLF, respectively) [21].

According to a recent systematic review that included 30 cohort studies with more than 43.000 patients with ACLF worldwide, the global prevalence of ACLF among acute hospitalized patients is around 35%, with an overall 90-day mortality of 58% [61]. Alcohol was the most frequently reported etiology of underlying cirrhosis (45%), infection was the most frequent trigger (35%) and kidney dysfunction the most common organ failure (49%) [61].

The clinical evolution of ACLF is very dynamic and comes to resolution, improvement or worsening (until death) in a short period of time [21,62]. More than its initial severity, the clinical course after 3-7 days from diagnosis better predicts the outcome. Therefore, a reassessment of ACLF grade and CLIF-C OF and CLIF-C ACLF scores within this time frame enables to stratify patients by potential outcome and monitor their response to treatments. These findings may have practical implications. Not surprisingly, patients with grade 3 ACLF after 3-7 days from diagnosis showed the worst prognosis. However, this group of extremely severe patients could be further stratified according to the number of concomitant organ failures. Indeed, 28-day transplant-free survival was 47% in patients with 3 failing organs but dropped to less than 10% in those with 4 or more concomitant organ failures. For the latter, if liver transplantation is contraindicated or not available, intensive support should be discontinued owing to futility [62].

1.3.3. Pathophysiological mechanisms underlying ACLF development

The pathophysiological mechanisms underlying the development of ACLF are still largely unknown. Evidence suggests that systemic inflammation represents both the underlying background and the main effector. Indeed, patients with ACLF present intense systemic inflammation and oxidative stress, different from patients who present acute decompensation without organ failure. Moreover, systemic inflammation directly correlates with the severity of ACLF: the greater the intensity of systemic inflammation, the larger the number of organ failures and the higher the short-term mortality [20,63,64].

From a mechanistic perspective, ACLF represent the condition in which all the above mentioned pathophysiological mechanisms reach their maximum extent, as a burst of systemic inflammation [20,23]. We already reported details about the role of PAMPs and DAMPs in overstimulating innate immune cells. Following this abnormal and sustained activation, different (and not mutually exclusive) mechanisms unfold their effects. Systemic inflammation can sustain and worsen effective hypovolemia and homeostatic compensatory mechanisms, ultimately leading to renal failure [4]. Detrimental effects due to the excessive immune activation cause tissue damage (immunopathology), leading to organ failure and sustaining DAMPs release [23,65]. Finally, in peripheral organs, systemic inflammation can induce mitochondrial dysfunction with impaired ATP production: it inhibits the translocation of fatty acids into the mitochondria, their beta-oxidation, and the electron transport chain, thus leading to the accumulation of free fatty acids and reactive oxygen species [33,34].

Notably, evidence also suggests that patients with ACLF present a defective host resistance to infections, a condition defined "immune paralysis" and whose precise mechanisms are still far to be completely understood. Along with the progression of ACLF and the burst of pro-inflammatory mediators, an excessive anti-inflammatory response also occurs (as showed by elevated circulating levels of IL-10 and IL-1ra) [20]. Moreover, studies demonstrated a defective response by macrophages and monocytes to PAMPs stimulation, and defective neutrophils production of antimicrobial superoxide anion and bactericidal activity [64,66–68]. Collectively, these humoral and

immune cell alterations configure a condition of "exhaustion" of immune effectors, that favors the development of serious infections [63,65,69].

1.3.4. Management of ACLF: unmet needs

A comprehensive review of ACLF treatment and clinical management falls beyond the scope of this thesis. At the same time, it is important to discuss some general principles and present some unmet needs that deserves future research.

So far, a global treatment for ACLF syndrome *per se* does not exist. The clinical management of patients with ACLF aims to diagnose and treat the precipitating event and then provide supportive therapy for impaired or failed organs. Patients with multiple organ failure or who do not respond to standard therapy, should be referred to an intensive care unit with care overseen by physician who are experts in liver management. Several therapeutic recommendations are currently based on clinical practice or on studies involving critically-ill patients without cirrhosis [70]. Liver transplantation could represent an effective treatment for patients with ACLF, as demonstrated by several studies. Indeed, the 1-year survival after liver transplantation does not differ between ACLF patients with one or two organ failures, and those without ACLF. Even for the most severe patients with three or more organ failures, the 1-year post-transplantation survival rate may approach 80%, dropping to less than 20% among patients who do not undergo transplantation [71–73].

In our opinion, three approaches could deserve a special interest for future research. First, preventive strategies based on drugs or treatments targeting key pathophysiological mechanisms, with the aim to modify the clinical course of the disease and prevent the development of acute decompensation and ACLF. Some potential agents are currently under investigations [74]. Second, artificial and bioartificial extracorporeal liver support systems with the aim to improve transplant-free survival or provide a bridge to transplantation. Several devices have been developed in the last three decades, but so far available evidence did not prove their efficacy [75,76]. The results of currently ongoing multicenter RCTs are eagerly awaited. Third, since systemic inflammation is a major pathophysiological substrate of ACLF, the development of intervention able to reduce

inflammation without inducing immunosuppression is a challenging issue for the years to come. Potential candidates are under investigations (e.g., TLR4-antagonists, IL-22), but further research is needed before introducing them in clinical practice [65,77,78].

To address these open issues, a deeper understanding of ACLF pathophysiology is of utmost importance. Any potential therapeutic strategies could only rely on a more precise knowledge of mechanisms underlying the development of acute decompensation and organ failures. Since ACLF is a major cause of death and hospitalizations in patients with decompensated cirrhosis, treating or preventing ACLF would imply to improve survival, and quality of life. Therefore, patients with ACLF (or at risk of ACLF development) deserve to be ever more precisely characterized: we need to know their distinctive clinical features (and risk factors) to update clinical practice; to understand their pathophysiological mechanisms to develop tailored therapies or targeted approaches; to determine genetic predisposing traits (if any) to put in place preventive strategies.

2. RESEARCH PROGRAM

During the three years PhD Program, we conducted different studies to characterize patients with acutely decompensated cirrhosis and ACLF from different perspectives.

We first performed a clinical study, with the aim to identify clinical predictors of nosocomial ACLF (i.e., ACLF developed during a hospitalization) in a cohort of patients with cirrhosis admitted to hospital for acute decompensation [79]. I also spent six months at the headquarters of the European Foundation for the study of Chronic Liver Failure (EF-CLIF), in Barcelona, Spain, with a Marco Polo scholarship of the University of Bologna. Under the mentorship of Richard Moreau and Vicente Arroyo, I had the opportunity to collaborate with the EF-CLIF's team of statisticians and bioinformaticians, with the aim to find out some new tiles for the complex mosaic of ACLF pathophysiology. We performed a further analysis of the blood metabolomic dataset collected from the CANONIC cohort, with a special focus on amino acids [80]. Both these two studies have been already published in international peer-reviewed Journals [79,80].

As a last perspective, we performed a genome-wide association study (GWAS) on DNA samples collected from our cohort of hospitalized patients with acutely decompensated cirrhosis, already enrolled in the first clinical study, with the aim to uncover potential underlying genetic factors involved in ACLF onset, progression, and outcomes.

2.1 Predictors of nosocomial ACLF in patients with acutely decompensated cirrhosis

As already reported, ACLF has a great impact on individual clinical course and survival, and represents a heavy burden on healthcare systems [61]. Therefore, ACLF prevention would assume great relevance. Effective prevention, however, can only ensue if predicting factors are known and easily identifiable and this matter is still ill-defined in acute patients. Independent predictors for the development of ACLF have been identified in stable outpatients with cirrhosis [81], but insufficient information is available in those admitted to hospital because of acute decompensation who subsequently develop ACLF.

2.1.1. Aim of the study

Objective of the study was the identification of simple and rapidly acquirable predictors of nosocomial ACLF (i.e., ACLF episode occurring during the hospitalization for acute decompensation) able to stratify patients according to their risk of developing this complication [79].

2.1.2. Patients and methods

We designed a prospective observational study performed in two Italian hospitals, the S. Orsola-Malpighi University Hospital in Bologna, and the Infermi Hospital in Rimini. All consecutive patients with liver cirrhosis non-electively admitted to the participating hospitals were screened for enrollment within 36 hours from admission [79].

2.1.3. Synthesis of the main results

The study enrolled 516 patients with cirrhosis consecutively admitted to hospital with acutely decompensated disease. ACLF was present at the time of hospitalization in 106 (21%) cases. Thus, the study cohort included 410 (79%) patients who did not meet the criteria for ACLF diagnosis at the time of hospital admission. Overall, 59 (14%) patients developed ACLF during hospitalization (nosocomial ACLF) after a median of 7 (IQR 4-18; min 2, max 45) days.

Patients who developed nosocomial ACLF had a more severe liver disease, with higher values of the prognostic scores (Child-Pugh, MELD, MELD-Na and CLIF C-AD), lower hemoglobin levels, more severe alterations of liver function parameters (serum bilirubin, serum albumin, and INR values), and higher serum creatinine concentration. Markers of systemic inflammation were significantly higher in this group of patients.

Among the baseline parameters significantly associated to the development of nosocomial ACLF at univariate analysis, lower hemoglobin level, higher leukocyte count, and higher MELD score were identified as independent risk factors at the competing risk regression analysis considering liver transplant, death, and hospital discharge as competing events. Interestingly, the probability of developing nosocomial ACLF progressively increased in parallel with the worsening of each risk

factor. The identified predictors were categorized according to the optimal cut-off value associated to the highest Youden index at ROC curve analysis: 9.8 g/dL for hemoglobin, 5.6 x 10^9/L for leucocyte count and 13 points for MELD score. These values were used to estimate the cumulative incidence of nosocomial ACLF in patients presenting none, 1, 2 or 3 risk factors. Notably, all patients who did not present any risk factor remained free from ACLF, while the cumulative incidence of in-hospital ACLF significantly increased in parallel with the number of risk factors, being 6% (95% CI 3-11), 21% (95% CI 15-28) and 59% (95% CI 38-75) in patients with 1, 2 or 3 risk factors, respectively. In each group of patients, stratified according to the number of risk factors, the probability of developing nosocomial ACLF was higher in patients developing nosocomial infection, rising from 3 to 29%, 16 to 50% and 52% to 83% in patients with 1, 2 or 3 risk factors, respectively (Figure 3) [79].

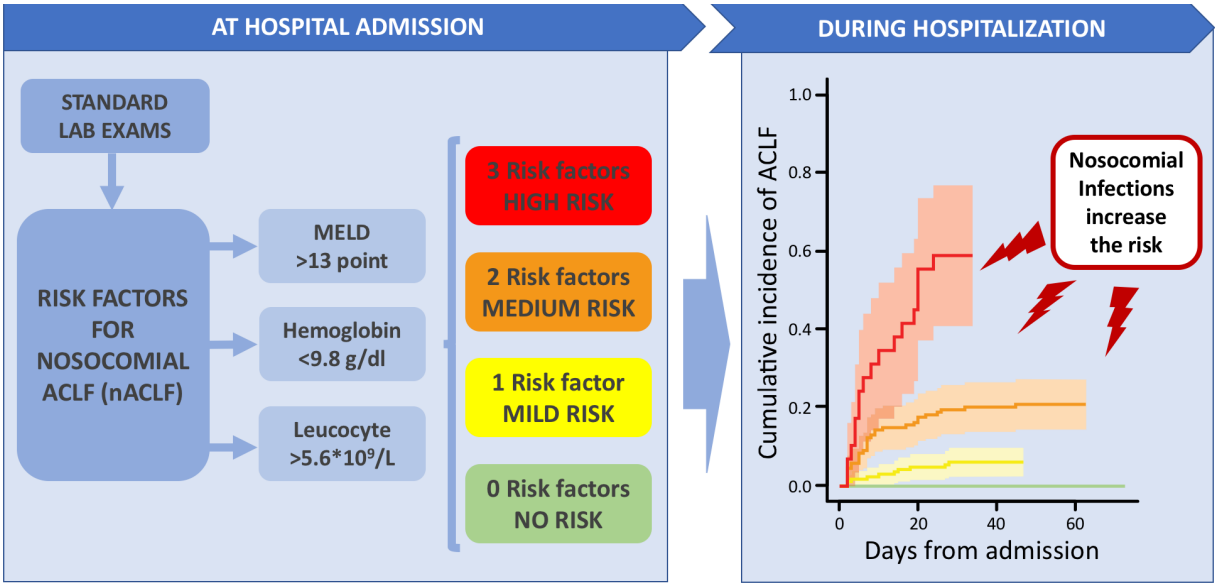


Figure 3. Graphical abstract of the published paper [79]. Standard laboratory exams at hospital admission allow to stratify patients according to their risk of developing ACLF during hospital stay. Patients at risk of developing this severe complication could require a strict clinical monitoring, in particular for the development of nosocomial infections.

2.1.4. Main findings and conclusions

In our study, nosocomial ACLF was independently predicted by MELD score, leukocyte count and hemoglobin level. That ACLF could be heralded by a greater severity of cirrhosis is an intuitive finding. More interesting were leukocyte count -in the absence of bacterial infection- and anemia. Leukocyte count is a rough but sensitive marker of systemic inflammation. It confirmed the

pathophysiological importance of a sustained "sterile" inflammation, deriving from the abnormal translocation of bacterial products from the gut (PAMPs) and the spread of molecules deriving from cell apoptosis and necrosis in the liver (DAMPs) [4,20,21]. Anemia also independently predicted the development of nosocomial ACLF. Indeed, the prognostic role of a reduced hemoglobin concentration in patients with cirrhosis has emerged sparsely in different settings [81–86]. It could be hypothesized that anemia may predispose to inflammation-induced peripheral organ dysfunction and, ultimately, failure by reducing tissue oxygen availability.

In conclusion, our analysis identified three easily available parameters (related to disease severity, systemic inflammation, and anemia), able to stratify patients with acutely decompensated cirrhosis into different, clear-cut levels of risk for the development of nosocomial ACLF. All of them are measurable shortly after admission to hospital or emergency department, thus enabling to put in place preventive measures and appropriate monitoring strategies. We acknowledge that our results would need validation in a different patient cohorts, but we think that it provided a solid background for further insights on this matter [79].

2.2 Changes in amino acids metabolisms in patients with ACLF

After the clinical study, we addressed some still unclear pathophysiological issues in collaboration with the EF-CLIF of Barcelona, Spain. At the headquarters of the Foundation, I had the opportunity to collaborate with a statistician and a bioinformatician, with the aim to perform a further analysis of the whole metabolomic dataset obtained by the CANONIC cohort [21,33,34].

We already reported how PAMPs and DAMPs can drive a sustained overactivation of immune system cells. The ultimate event of this cascade is multi-organ dysfunction and failure, but the precise intermediate mechanisms are still unclear. As mentioned above, several mechanisms have been demonstrated or at least hypothesized (worsening of arterial vasodilation with tissue hypoperfusion, microvascular dysfunction, direct tissue damage, mitochondrial dysfunction with cellular energetic crisis) but a comprehensive understanding is still far to be obtained (Figure 1).

How do amino acids get involved in ACLF metabolic changes? The immune system activation (like in sepsis) requires an anabolic, energy-consuming metabolism, resulting in synthesis of nucleotides, proteins and lipids that support leukocyte proliferation, and biosynthesis of biomolecules involved in host defense. Notably, patients with decompensated cirrhosis present sarcopenia (worsened during acute events and ACLF), indicating intense skeletal muscle catabolism. Circulating amino acids are released from internal stores and represent the building blocks for protein and nucleotides synthesis. The one carbon metabolic network (composed by the folate cycle and the methionine cycle) contributes to this process.

Therefore, available evidence suggests a potential role of several amino acids in contributing to the intense metabolic changes, already demonstrated in patients with ACLF [33,34]. The mechanistic link between amino acids metabolic pathways, systemic inflammatory response and organ failure development, is an open issue that we tried to address [80].

2.2.1. Aim of the study

We reanalyzed the blood metabolome data set obtained from the CANONIC cohort [33] with the aim to assess the potential role of major amino acid metabolic pathways in systemic inflammatory responses and organ failures, among patients with acutely decompensated cirrhosis, with or without ACLF. Because metabolisms of amino acids and glucose are interrelated, we used the entire metabolic data set to perform correlative and integrative analyses [80].

2.2.2. Patients and methods

The study cohort included 831 patients with acutely decompensated cirrhosis enrolled in the CANONIC study [21], 181 with ACLF and 650 with acute decompensation. The blood metabolomic dataset comprised 137 blood metabolites, identified using untargeted metabolomics by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) [33].

The analysis implied different steps. First, we assessed correlations between metabolites, irrespective of their nature, therefore capturing contextual changes in amino acid levels. We used

weighted gene co-expression network analysis (WGCNA), which has been originally developed to generate modules of correlated genes and then has been used to identify modules of correlated metabolites [87,88]. Then, for each module we computed an eigenmetabolite [89], as a representative value of the entire module that can be used for correlations with orthogonal data sets including clinical data, and inflammatory markers obtained in patients from the CANONIC cohort. As a second step, we calculated the fold-change of each metabolite in ACLF relative to AD. Finally, for each metabolite we manually queried PubMed (www.ncbi.nlm.nih.gov/pubmed), the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg), and the Human Metabolome Database (HMDB, www.hmdb.ca) aiming to chart metabolic pathways that could be differentially engaged between ACLF and AD, and examine their potential overlap with modules identified at the first step of analysis [80].

2.2.3. Synthesis of the main results

We found that significant correlations exist between metabolites of the entire data set: many metabolite pairs were linked by a positive and significant correlation and several metabolites were involved in more than one pair of significant correlations, suggesting the existence of groups of positively co-regulated metabolites. Therefore, using WGCNA, we identified 9 modules of co-regulated metabolites; each module received a color name, arbitrarily given by the algorithm. Metabolites related to amino acids represented 43% of the metabolic data set and were the only category to be present in each of the 9 modules. Amino acids and related molecules were exclusive members of the yellow module and represented most metabolite members of the pink and magenta modules (86% and 80%, respectively).

Comparing eigenmetabolite values (one per module) between patients with ACLF and those without, we found that each eigenmetabolite was significantly higher in patients with ACLF, consistent with a prominent upregulation of the blood metabolome [33], and the increase paralleled ACLF severity (assessed by CLIF-C OF and ACLF grade). We then assessed the correlation of the 9 eigenmetabolites with well-known markers of systemic inflammation and oxidative stress: most

modules were positively and significantly correlated, consistent with a metabolite module upregulation in parallel with both systemic inflammation and oxidative stress. Again, many modules were positively and significantly correlated with liver, kidney, and circulatory dysfunction. Notably, we demonstrated that metabolite accumulation only partially depends on a mere reduction of renal excretion, but it is the result of several independent effects.

Module upregulation preceded major patient outcomes, such as ACLF development and death. Indeed, among patients without ACLF at admission, 8 out of 9 eigenmetabolites were significantly higher in those that developed ACLF by 28 days. Moreover, the same eigenmetabolites were increased in patients who died within 28 days, compared to those who survived.

In patients with ACLF, we found a network of co-orchestrated modules (yellow-blue-brown-green) that included metabolites related to aerobic glycolysis, pentose phosphate pathway, Krebs cycle intermediates, folate cycle, methionine salvage pathway, and purine and pyrimidine synthesis. Moreover, accumulated metabolites showed an engagement of methionine cycle and transsulfuration pathway. Finally, we found an accumulation of fatty acids and acylcarnitines (consistent with a suppression of fatty acids beta-oxidation and ATP synthesis in the mitochondria) and of ketogenic amino acids, suggesting a defective ketone body production.

2.2.4. Main findings and conclusions

Blood metabolome changes in patients with ACLF are characterized by prominent metabolite accumulation, and metabolic changes paralleled the intensity of systemic inflammation and the severity of ACLF. This upregulation precedes the occurrence of severe clinical outcomes, thus suggesting that blood metabolites should be evaluated as biomarkers in future studies.

Amino acids accumulation could be probably explained by their mobilization from skeletal muscle, with the aim to fuel *de novo* protein synthesis in cells involved in innate immune response. Moreover, features of increased glycolysis (and PPP), extraction of Krebs cycle intermediates and nucleotide synthesis, could also be explained by the necessity to fuel immune activation. We also found features suggesting the activation of the transsulfuration pathway (a branch of the

methionine cycle that gives rise to the antioxidant glutathione), a finding that makes sense in patients with ACLF, where systemic oxidative stress develops [20,23]. The polyamine spermidine (a product of methionine and ornithine metabolism) showed decreased blood levels in ACLF, probably because of a polyamine skewing towards GABA synthesis. Decreased levels of spermidine (an inducer of autophagy) may play a role in the decrease in autophagy, found in ACLF [90,91].

In conclusion, our results suggested that, in ACLF, amino acids may be mobilized from skeletal muscles or derived from Krebs cycle intermediates to fuel anabolic programs, including protein and nucleotide synthesis, engaged by intense innate immune responses. Ketogenic amino acids were extensively catabolized to produce energy substrates in peripheral organs, an effect that was insufficient, leading to organ failures. Finally, the polyamine spermidine which is a potent inducer of autophagy with anti-inflammatory effects, had decreased blood levels in ACLF [80]. The main limitation of our study was its descriptive design. Our hypotheses were not challenged by mechanistic investigations in animal models or in patients with acute systemic inflammation (e.g., sepsis) without cirrhosis. These remain open issues that deserve future investigations.

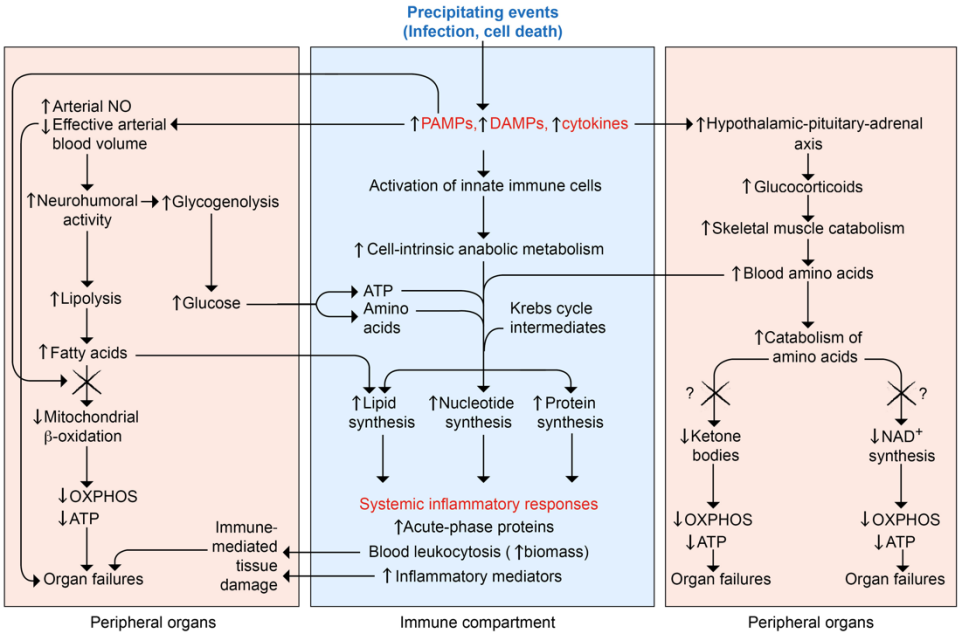


Figure 4. Graphical abstract of the published paper [80]. In ACLF, skeletal muscle catabolism occurs resulting in increased circulating amino acids that are used to fuel protein and nucleotide synthesis, required by intense systemic inflammatory responses. Ketogenic amino acids were extensively catabolized to produce energy substrates in peripheral organs, an effect that is not sufficient thus leading to organ failure. See text for further details. NO: nitric oxide; PAMPs: pathogen-associated molecular patterns; DAMPs: damage-associated molecular patterns; ATP: adenosine triphosphate; NAD: nicotinamide adenine dinucleotide; OXPHOS: oxidative phosphorylation.

2.3 A third perspective: genetic factors underlying acutely decompensated cirrhosis

As the final step of our research activities during the PhD Program, we addressed acutely decompensated cirrhosis and ACLF from another perspective. We performed a genome-wide association study (GWAS) on DNA samples from our cohort of non-electively hospitalized patients enrolled in the first clinical study, to uncover potential underlying genetic factors involved in ACLF onset, progression, and outcomes.

To do that, we established an important collaboration with the research group of Prof. Paolo Garagnani, from the Department of Experimental, Diagnostic and Specialty Medicine (DIMES) of the University of Bologna, that supervised some of the analyses.

3. GENOME-WIDE ASSOCIATION STUDY

3.1 Background

As widely described in previous chapters, ACLF development is a major complication of cirrhosis and represents one of the main causes of hospitalization and death for patients with decompensated disease. Several studies addressed clinical characteristics and predictors of ACLF, and growing evidence is progressively uncovering its pathophysiological mechanisms, that are closely related to a setting of severe systemic inflammation and immune system activation.

On the other hand, only scant information is available on genetic factors potentially involved in individual susceptibility, occurrence, and outcome of ACLF episodes. Indeed, only few studies are available on this matter. Two of them assessed the association of specific polymorphisms, namely for interleukin (IL)-1 gene cluster and for innate immune components, with the severity of systemic inflammation or with clinical outcomes in patients with ACLF and bacterial infections included in the CANONIC cohort [92,93]. Other studies, mainly conducted in China, assessed the association between genetic factors and clinical traits of HBV-related ACLF [94–96]. Notably, this clinical condition is a very specific type of acute hepatic impairment/failure: it is paradigmatic of ACLF episodes in Asia [47], but almost negligible in Western countries. Moreover, HBV-related chronic liver disease is a very specific setting, that involves peculiar immunological mechanisms related to chronic persistence of HBV infection, and HBV-related advanced liver diseases [97].

Genome-wide association study (GWAS) is a systematic, well-powered, unbiased, genome-wide survey exploring the relationships between common sequence variation and disease predisposition, which has improved our understanding of the genetic basis of many complex traits [98]. Different from candidate gene-based studies, GWAS test hundreds of thousands to millions of common single nucleotide polymorphisms (SNPs) across the genome, providing an unbiased method to investigate genetic risk loci, and allowing the discovery of novel disease-relevant genes.

So far, no studies performed GWAS in acutely decompensated patients with and without ACLF, as defined by the EASL-CLIF criteria. Therefore, we decided to address this issue.

3.2 Aim of the study

The objective of the study was to perform an untargeted exploration of genetic variants associated with major clinical traits and outcomes of patients with acutely decompensated cirrhosis, to assess any potential relationship between genetic factors and clinical phenotypes.

3.3 Patients and methods

3.3.1. Study population

The study population included subjects enrolled in a prospective observational study performed in two Italian hospitals, the S. Orsola-Malpighi University Hospital in Bologna, and the Infermi Hospital in Rimini. The study protocol was approved by the local institutional review boards. Written informed consent was obtained from patients or from legal surrogates before enrollment, according to the 1975 Declaration of Helsinki.

As already reported for the clinical study, all consecutive patients with liver cirrhosis admitted to the participating hospitals were screened for enrollment by an experienced Hepatologist within 36 hours from admission. Inclusion criteria were a) cirrhosis diagnosed by a composite of clinical signs, laboratory tests, endoscopy, and imaging; b) admission because of an acute decompensating event; c) age >18. Exclusion criteria were a) elective admission for a scheduled procedure; b) hepatocellular carcinoma beyond the Milan criteria [99]; c) severe chronic extrahepatic diseases; d) extrahepatic malignancy; e) previous liver transplantation.

During the hospitalization, all patients were assessed daily for the development of bacterial infections and ACLF. The attending physician oversaw patient clinical management according with international and local guidelines. After hospital discharge, patients were prospectively followed up to 1 year and data on liver transplantation, death, and causes of death were collected.

3.3.2. Design of the analysis

To avoid an excessive number of patient subgroups thus exceeding in granularity and loosing significance in the association with genetic data, we arbitrarily decided to define 2 groups of

patients, based on major clinical features, and further divide each of them in 2 subgroups according to the 1-year survival status. The first group included patients that presented (at enrollment or during the hospital stay) an episode of ACLF or bacterial infection, well-known major clinical events for patients with cirrhosis. We associated these two events for their strict connection, both from a clinical and a pathophysiological point of view. The second group included patients that remained free from both. For each group, we defined 2 subgroups according to the mortality status after 1 year of follow up. Therefore, we obtained and compared 4 groups of patients. We are aware that this choice may seem an excessively gross classification, but it was made with the aim to define clear-cut groups based on the combination of major clinical traits and hard outcomes.

Subsequently, we performed two main comparisons. In the first, patients presenting the most severe clinical features and outcomes (with ACLF or bacterial infections, and dead within 1 year) were compared to patients with the mildest clinical course (free of ACLF and bacterial infections and alive at 1 year). In the second, we assessed patients presenting the most severe clinical traits (ACLF or bacterial infections during hospitalization) but with distinct clinical outcomes: those who died within 1 year compared to those who survived.

3.3.3. Data collection and definitions

Data were collected using an online electronic case report form and their integrity was systematically checked before being entered into the database. At the time of enrollment were collected the following data: demographic characteristics, etiology of cirrhosis, laboratory and clinical features including the presence of hepatocellular carcinoma (HCC) and/or other co-morbidities assessed by the Charlson score [100]. The Model for End-stage Liver Disease (MELD) [101], MELD-sodium [102], Child-Turcotte-Pugh [103], CLIF-C OF [59], CLIF-C Acute Decompensation (CLIF-C AD) [104] and CLIF-C ACLF [59] scores were calculated.

Acute decompensation of cirrhosis was defined by a) acute onset of grade 2 or 3 ascites, according to the International Club of Ascites classification [105]; b) new episode of hepatic encephalopathy graded according to the West-Haven criteria [106] in patient with previous normal

consciousness and no evidence of an acute neurologic disease; c) upper or lower gastrointestinal bleeding; d) bacterial infection; e) any combination of these events. ACLF was diagnosed and graded according to the EASL-CLIF criteria [21]. Bacterial infections were diagnosed according with international and local guidelines [107–109]. An experienced Infectious Disease specialist revised each event of infection for consistency and accuracy.

3.3.4. Statistical analysis

In the first phase of the analysis, we described the enrolled population and compared different patient subgroups according to their clinical and laboratory parameters. For all continuous parameters the normality of distribution and homogeneity of variance were evaluated by the Kolmogorov-Smirnov and Levene tests, then variables were reported as mean and standard deviation or median and interquartile range (IQR) as appropriate. Accordingly, comparisons between groups were performed by means of Student's t Test or Mann-Whitney U test when appropriate. Categorical parameters were reported as frequency and percentage and compared by the $\chi 2$ square test. All tests were two-sided and p values <0.05 were considered statistically significant. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 25, IBM corp) and the cmprsk package on R statistical software (http://www.R-project.org/).

As a second step, we genotyped the collected DNA samples to determine SNPs significantly associated with clinical outcomes and patient subgroups. Genotype data were acquired using Illumina™ Infinium CoreExome-24 v1.4 BeadChip microarray. All the preprocessing steps involving filtering of samples and SNPs were performed with PLINK toolset version 1.07. The sex assignment was first verified and all samples for which clinical information on sex and the number of X chromosomes in whole genome sequencing (WGS) data did not match, or with a failed genotype-based sex prediction, were excluded. Samples with missing genotype rate above 10% also were not considered in further analysis. Filtering of SNPs excluded all the variants that: a) had low minor allele frequency (MAF < 0.03), b) reached missing genotyping rate above 10%, c) failed the test for Hardy-Weinberg Equilibrium (p-value < 0.001). WGS data cleaning and association analysis were

performed in R environment version 3.6.3. Principal component analysis (PCA) was applied to detect outliers; consequently, individuals that were not of Caucasian race, and samples with |z-scores| for two principal components above 3 were excluded. SNPRelate R package was used to identify and remove redundant correlated SNPs that were in linkage disequilibrium (0.2 used as LD threshold), and possible related samples (0.1 used as kinship coefficient threshold). Association analysis was performed fitting generalized linear model for each SNP and adjusting the nominal p-value for multiple tests with Bonferroni's method. For the variants that after correction reached the statistical significance level of 0.05, quantile-quantile plots and lambda entity values were controlled to exclude the presence of stratification.

Finally, after the identification of the SNPs significantly associated with clinical outcomes and patient subgroups, we manually queried UCSC Genome Browser (genome.ucsc.edu), ENCODE (www.encodeproject.org), GeneCards - The Human Gene Database (https://www.genecards.org/), and PubMed (www.ncbi.nlm.nih.gov/pubmed) with the objective to chart genes and related transcripts or products with potential clinical implications.

3.4 Results

3.4.1. Study population

We collected DNA samples from 319 patients out of the 516 patients with acutely decompensated cirrhosis enrolled in the clinical study, as previously described. Among the available samples, 41 were subsequently excluded after a DNA quality control (samples with missing genotype >10%). Moreover, 5 individuals that were not of Caucasian race, and 3 outliers (samples with |z-scores| for two principal components above 3) were also excluded. Thus, the genotyped population of the GWAS included 270 patients, as reported in Figure 5.

Among the enrolled patients, 156 presented ACLF or bacterial infection, at admission or during hospital stay; 76 of them died within 1 year (group 1), and the remainder 80 survived (group 2). Among the 114 subjects that did not develop ACLF or bacterial infections, 38 died by 1-year follow up (group 3), and 76 survived (group 4). Figure 5 reports patient disposition.

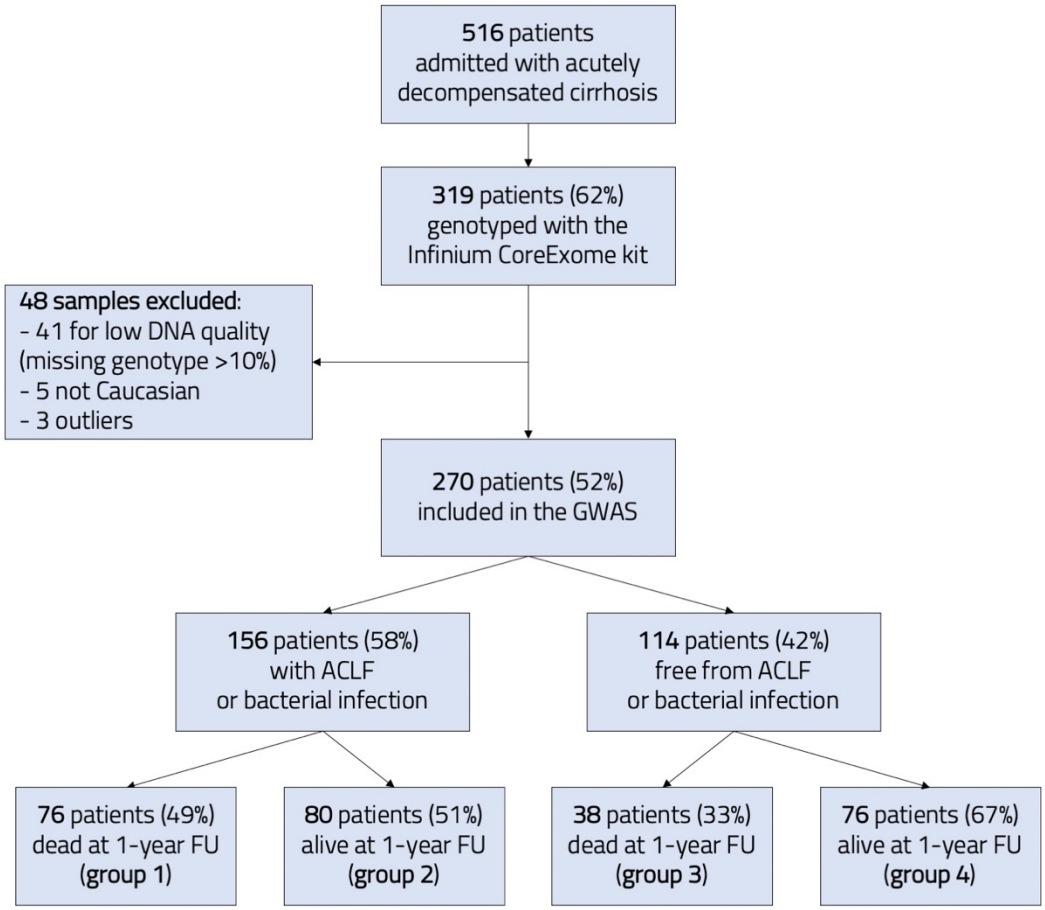


Figure 5. Patient disposition. Genotyped patients with acutely decompensated disease and available high-quality DNA samples were 270. We divided them in four groups, according to the presence of severe clinical events (ACLF or bacterial infections) and subsequently according to the 1-year survival status. See text for details. GWAS: genome-wide association study; FU: follow up.

3.4.2. Baseline characteristics of genotyped patients and groups

Baseline demographic, laboratory and clinical data of genotyped patients included in the GWAS are reported in Table 1. The median age was 64 years and male sex was more prevalent; the median values of MELD and MELD-Na scores were 16 and 19, respectively, and patients were mainly in B class of Child-Pugh score. Ascites was the most frequent decompensating event. Overall, ACLF was diagnosed in 98 (36%) patients, 70 (26%) of them at hospital admission, while in the remainder 28 (10%) during hospital stay, consistently with the main international cohorts [21,61]. Moreover, 67 patients presented bacterial infections at hospital admission, while 38 cases of nosocomial infections occurred during hospitalization.

Table 1. Baseline demographic, biochemical and clinical characteristics of genotyped patients.

	All patients (n=270)
Demographic data	
Age (years)	64 (53 - 75)
Male sex	170 (63%)
Etiology of cirrhosis	
Viral	129 (48%)
Alcohol	49 (18%)
NASH	21 (8%)
Other or mixed etiology	71 (26%)
Hematology, biochemistry, and prognostic scores	
Hemoglobin (g/dL)	10.6 (9.4 - 12.1)
Leucocytes (10^9/L)	5.81 (4.06 - 9.17)
CRP (mg/dL)	1.24 (0.49 - 3.78)
Platelets (10^9/L)	92 (59 - 149)
Sodium (mmol/L)	136 (133 - 139)
Bilirubin (mg/dL)	2.44 (1.21 - 5.06)
Creatinine (mg/dL)	1.08 (0.79 - 1.5)
INR	1.37 (1.24 - 1.63)
Albumin (g/dL)	3.1 (2.8 - 3.5)
MELD score	16 (11 - 22)
MELD-Na score	19 (13 - 24)
Child-Pugh score	9 (7 - 11)
Clinical data	
Ascites	174 (64%)
Encephalopathy III/IV	54 (20%)
Renal dysfunction	65 (24%)
Gastrointestinal bleeding	18 (7%)
Community acquired bacterial infection	42 (16%)
Health care related bacterial infection	25 (9%)
Hospital acquired bacterial infection	38 (14%)
ACLF at admission	70 (26%)
ACLF during hospitalization	28 (10%)
HCC	70 (26%)
Diabetes	96 (36%)
28-day death status	29 (11%)
90-day death status	61 (23%)
180-day death status	90 (33%)
365-day death status	114 (42%)

NASH: non-alcoholic steatohepatitis; CRP: C-reactive protein; INR: international normalized ratio; MELD: model for end-stage liver disease; MELD-Na: MELD incorporating serum sodium; CLIF-C AD: Chronic Liver Failure Consortium acute decompensation score; HCC: hepatocellular carcinoma.

We then performed a comparison of baseline parameters between patients of group 1 and group 4 (patients with the most severe and the mildest clinical course, respectively), as reported in table 2. Patients that developed ACLF or infection and died within 1 year (group 1) were older, showed higher markers of systemic inflammation (leukocyte count and CRP) and a more severe liver disease (more severe alterations of prognostic scores and parameters of liver function). Moreover, they presented more frequently ascites, hepatic encephalopathy, and renal dysfunction.

Table 2. Baseline demographic, biochemical and clinical characteristics of groups 1 and 4.

	Group 1 (n=76)	**Group 4 (n=76)**	**P value**
Demographic data			
Age (years)	67 (56 - 77)	61 (50 - 73)	0.015
Male sex	45 (59%)	46 (61%)	1.000
Etiology of cirrhosis			
Viral	39 (51%)	37 (49%)	0.871
Alcohol	10 (13%)	8 (11%)	0.803
NASH	5 (7%)	5 (7%)	1.000
Other or mixed etiology	22 (29%)	26 (34%)	0.601
Hematology, biochemistry, and prognostic scores			
Hemoglobin (g/dL)	10.53±2.16	11.09±2.35	0.129
Leucocytes (10^9/L)	8.56 (5.4 - 10.66)	4.98 (3.33 - 6.91)	<0.001
CRP (mg/dL)	2.76 (1.02 - 7.27)	0.56 (0.22 - 1.3)	<0.001
Platelets (10^9/L)	88.5 (53 - 149.5)	105 (57.5 - 161)	0.379
Sodium (mmol/L)	136 (133 - 141)	138 (136 - 140)	0.151
Bilirubin (mg/dL)	3.87 (1.93 - 9.14)	1.71 (0.9 - 3.2)	<0.001
Creatinine (mg/dL)	1.42 (0.98 - 2.09)	0.8 (0.7 - 1.09)	<0.001
INR	1.51 (1.32 - 1.92)	1.3 (1.18 - 1.43)	<0.001
Albumin (g/dL)	3 (2.7 - 3.4)	3.28 (2.91 - 3.57)	0.016
MELD score	22 (16 - 28)	11 (9 - 15)	<0.001
MELD-Na score	24 (19 - 30)	13 (11 - 17)	<0.001
Child-Pugh score	9 (8 - 11)	7 (6 - 9)	<0.001
Clinical data			
Ascites	56 (74%)	41 (54%)	0.018
Encephalopathy III/IV	22 (29%)	11 (15%)	0.048
Renal dysfunction	31 (41%)	4 (5%)	<0.001
Gastrointestinal bleeding	2 (3%)	8 (11%)	0.098
Bacterial infection (any)	53 (70%)	0 (0%)	<0.001
ACLF	56 (74%)	0 (0%)	<0.001

NASH: non-alcoholic steatohepatitis; CRP: C-reactive protein; INR: international normalized ratio; MELD: model for end-stage liver disease; MELD-Na: MELD incorporating serum sodium.

As a second comparison, we considered groups 1 and 2, i.e., patients who developed bacterial infections or ACLF, and died within 1 year (group 1) or survived (group 2). Again, patients from group 1 were older and showed a higher leukocyte count (but not CRP levels). Parameters of liver function (bilirubin and INR), and consequently the main prognostic scores (MELD and MELD-Na) were slightly worse, compared to group 2. Prevalence of bacterial infections and ACLF were not significantly different, except for nosocomial ACLF (higher in group 1).

Table 3. Baseline demographic, biochemical and clinical characteristics of groups 1 and 2.

	Group 1 (n=76)	**Group 2 (n=80)**	**P value**
Anthropometric data			
Age (years)	67 (56 – 77)	59 (53 - 67)	0.002
Male sex	45 (59%)	59 (74%)	0.063
Etiology of cirrhosis			
Viral	39 (51%)	32 (40%)	0.198
Alcohol	10 (13%)	20 (25%)	0.070
NASH	5 (7%)	10 (13%)	0.280
Other or mixed etiology	22 (29%)	18 (23%)	0.367
Hematology, biochemistry, and prognostic scores			
Hemoglobin (g/dL)	10.53±2.16	10.49±1.78	0.902
Leucocytes (10^9/L)	8.56 (5.4 – 10.66)	6.32 (4.28 - 9.4)	0.025
CRP (mg/dL)	2.76 (1.02 - 7.27)	1.94 (0.69 - 3.97)	0.089
Platelets (10^9/L)	88.5 (53 - 149.5)	92.5 (63.5 - 144)	0.702
Sodium (mmol/L)	136 (133 – 141)	136 (133 - 139)	0.218
Bilirubin (mg/dL)	3.87 (1.93 - 9.14)	2.39 (1.22 - 7.11)	0.041
Creatinine (mg/dL)	1.42 (0.98 - 2.09)	1.2 (0.9 - 1.72)	0.101
INR	1.51 (1.32 - 1.92)	1.39 (1.23 - 1.66)	0.048
Albumin (g/dL)	3 (2.7 - 3.4)	3.1 (2.8 - 3.5)	0.173
MELD score	22 (16 - 28)	18 (13 - 24)	0.005
MELD-Na score	24 (19 - 30)	21 (16 - 27)	0.021
Child-Pugh score	9 (8 - 11)	9 (7 - 11)	0.014
Clinical data			
Ascites	56 (74%)	47 (59%)	0.063
Encephalopathy III/IV	22 (29%)	11 (14%)	0.030
Renal dysfunction	31 (41%)	30 (38%)	0.743
Gastrointestinal bleeding	2 (3%)	7 (9%)	0.168
Bacterial infection (any)	53 (70%)	52 (65%)	0.572
ACLF at admission	37 (49%)	33 (41%)	0.421
ACLF during hospitalization	19 (25%)	9 (11%)	0.036

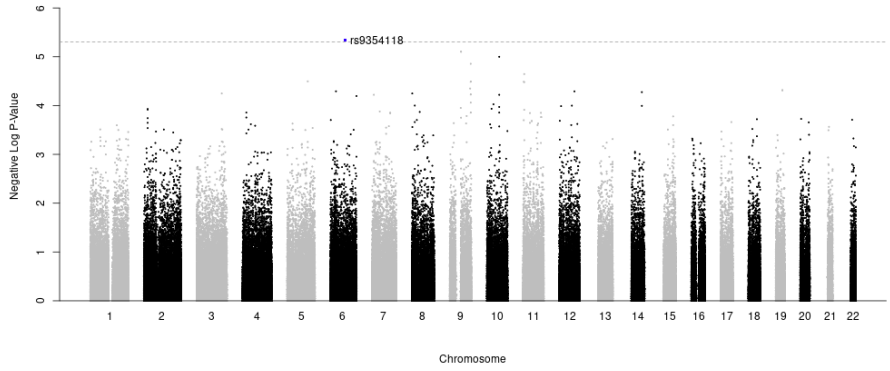
NASH: non-alcoholic steatohepatitis; CRP: C-reactive protein; INR: international normalized ratio; MELD: model for end-stage liver disease; MELD-Na: MELD incorporating serum sodium.

3.4.3. GWAS data

In the initial GWAS stage, we genotyped 547644 SNPs across the genome in 270 patients, as already described. After stringent quality controls and filtering steps, 268117 SNPs were retained for further analyses.

Our genotype-phenotype association analysis suggested that 3 SNPs were associated with the selected clinical phenotypes (Figure 6). Indeed, rs9354118 was associated with the *worst* clinical phenotype (group 1: presence of ACLF/infections, and death during follow up) compared to the *mildest* (group 4: patients free from ACLF/infections and alive at 1 year). On the other hand, rs1146878 and rs6479397 were associated with death within 1 year among patients that presented ACLF or infections during hospitalization (group 1 vs group 2).

Comparison 1 (group 1 vs group 4)



Comparison 2 (group 1 vs group 2)

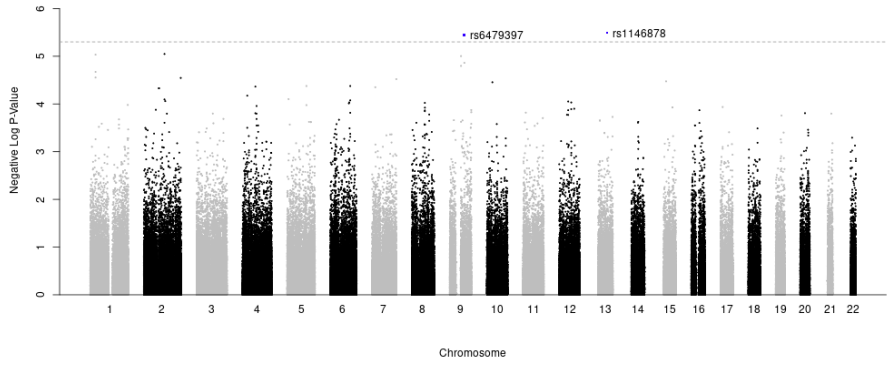


Figure 6. Signal–intensity (Manhattan) plots of the genome-wide p values of the genotyped SNPs. **Upper panel**: comparison 1 (group 1 vs group 4). **Lower panel**: comparison 2 (group 1 vs group 2).

Polymorphisms rs9354118, rs1146878, and rs6479397 are located on chromosome 6q16.1, 13q22.2 and 9q22.31, respectively. They belong to non-codifying regions, although they are near to (within 2 kb, as in case of rs9354118) or within (as for rs1146878, rs6479397) a known candidate Cis-Regulatory Element (cCRE), potentially involved in the regulation of nearby gene expression. We then manually queried UCSC Genome Browser (genome.ucsc.edu), ENCODE (www.encodeproject.org), GeneCards - The Human Gene Database (https://www.genecards.org/), and PubMed (www.ncbi.nlm.nih.gov/pubmed) aiming to chart neighboring genes and related transcripts with potential clinical implications (Table 4).

Table 4. Synthesis of the available information about transcripts and known functions of nearby genes of the identified significant SNPs. In bold, features of interest for patients with ACLF.

SNP: **rs9354118** (chr 6q16.1)

Genes	Aliases	Synthesis of known roles and functions
EPHA7	EPH Receptor A7 Ephrin Type-A Receptor 7	Receptor tyrosine kinase which binds promiscuously GPI-anchored ephrin-A family ligands residing on adjacent cells, leading to contact-dependent bidirectional signaling into neighboring cells. Forward signaling may result in activation of components of the ERK signaling pathway including MAP2K1, MAP2K2, MAPK1 AND MAPK3 which are phosphorylated upon activation of EPHA7. Among its related pathways are Actin Nucleation by ARP-WASP Complex and Activation of cAMP-Dependent PKA.
MANEA	Mannosidase Endo-Alpha Endomannosidase	MANEA catalyzes the release of mono-, di-, and triglucosylmannose oligosaccharides by cleaving the alpha-1,2-mannosidic bond that links them to high-mannose glycans. Endomannosidase is involved in N-glycosylation of proteins in the endoplasmic reticulum.
FUT9	Fucosyltransferase 9	It belongs to the glycosyltransferase family and catalyzes the last step in the biosynthesis of glycan determinant Lewis X (LeX/CD15), the addition of a fucose to precursor polysaccharides. **It is responsible for the expression of CD15 in mature granulocytes. A common haplotype of this gene has been associated with susceptibility to placental malaria infection. CD15 is a distinguishing marker for human myeloid cells and mediates neutrophil adhesion to dendritic cells.**
UFL1	UFM1 Specific Ligase 1 E3 Protein Ligase 1	E3 protein ligase that mediates ufmylation, the covalent attachment of the ubiquitin-like modifier UFM1 to lysine residues on target proteins, and which plays a key role in reticulophagy, induced in response to endoplasmic reticulum stress. **Ufmylation in response to endoplasmic reticulum stress is essential for processes such as hematopoiesis, blood vessel morphogenesis or inflammatory response. It regulates inflammation in response to endoplasmic reticulum stress by promoting reticulophagy, leading to inhibit the activity of the NF-kappa-B transcription factor**. Also involved in the response to DNA damage: recruited to double-strand break sites following DNA damage and mediates mono-ufmylation of histone H4.

SNP: **rs1146878** (chr 13q22.2)

Genes	Aliases	Known roles and functions
LMO7	LIM Domain 7	This gene encodes a protein containing a calponin homology (CH) domain, a PDZ domain, and a LIM domain, and may be involved in protein-protein interactions. **It is involved in Class I MHC mediated antigen processing and presentation, a fundamental process of innate immune system (NK cells)**.
KCTD12	Potassium Channel Tetramerization Domain Containing 12	Auxiliary subunit of GABA-B receptors that determine the pharmacology and kinetics of the receptor response. Increases agonist potency and markedly alter the G-protein signaling of the receptors by accelerating onset and promoting desensitization. Among its related pathways are the activation of cAMP-dependent PKA and hepatic ABC transporters, related to cholestatic diseases.

ACOD1	Cis-Aconitate Decarboxylase	Cis-aconitate decarboxylase catalyzes production of itaconate and **is involved in the inhibition of the inflammatory response. Acts as a negative regulator of the Toll-like receptors (TLRs)-mediated inflammatory innate response by stimulating the tumor necrosis factor alpha-induced protein TNFAIP3 expression via reactive oxygen species (ROS) in LPS-tolerized macrophages. Involved in antimicrobial response of innate immune cells; ACOD1-mediated itaconic acid production contributes to the antimicrobial activity of macrophages**.
CLN5	Ceroid-Lipofuscinosis Neuronal Protein 5	This gene is one of eight which have been associated with neuronal ceroid lipofuscinoses (NCL). Also referred to as Batten disease, NCL comprises a class of autosomal recessive, neurodegenerative disorders affecting children. The primary defect in NCL disorders is thought to be associated with lysosomal storage function.
FBXL3	F-Box and Leucine-rich Repeat Protein 3	This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. It is a component of the SCF(FBXL3) E3 ubiquitin ligase complex involved in circadian rhythm function. The SCF(FBXL3) complex mainly acts in the nucleus and mediates ubiquitination and subsequent degradation of CRY1 and CRY2.
MYCBP2	MYC Binding Protein 2	The encoded protein plays a role in axon guidance and synapse formation in the developing nervous system. In mammalian cells, this protein regulates the cAMP and mTOR signaling pathways, and may additionally regulate autophagy.

SNP: **rs6479397** (chr 9q22.31)

ROR2	Receptor Tyrosine Kinase-like Orphan Receptor 2	Tyrosine-protein kinase receptor which may be involved in the early formation of the chondrocytes. It seems to be required for cartilage and growth plate development. Phosphorylates YWHAB, leading to induction of osteogenesis and bone formation
SPTLC1	Serine Palmitoyl-transferase Long Chain Base Subunit 1	This gene encodes a member of the class-II pyridoxal-phosphate-dependent aminotransferase family. Serine palmitoyltransferase converts L-serine and palmitoyl-CoA to 3-oxosphinganine with pyridoxal 5'-phosphate and is the key enzyme in sphingolipid biosynthesis. Mutations in this gene were identified in patients with hereditary sensory neuropathy type 1.
PRSS47	Putative Serine Protease 47	Serine Protease 47 is related to serine-type endopeptidase activity. There is disagreement about whether it encodes a protein or is a pseudogene.
IARS1	Isoleucyl-TRNA Synthetase 1	Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNA. Because of their central role in linking amino acids with nucleotide triplets contained in tRNAS, aminoacyl-tRNA synthetases are thought to be among the first proteins that appeared in evolution. Isoleucine-tRNA synthetase belongs to the class-I aminoacyl-tRNA synthetase family and has been identified as a target of autoantibodies in the autoimmune disease polymyositis/dermatomyositis.

As highlighted in bold font in Table 4, some of the neighboring genes express proteins with functions of potential great interest in patients with cirrhosis and ACLF, based on the pathophysiological background that we extensively mentioned in previous chapters. This is the case of FUT9 and UFL1 for SNP rs9354118, and LMO7 and ACOD1 for SNP rs1146878 (Table 4).

3.5 Discussion and conclusions

To our knowledge, only scarce information is currently available on genetic factors involved in clinical phenotypes of acutely decompensated cirrhosis and ACLF. Moreover, our study is the first untargeted GWAS conducted on a large cohort of hospitalized patients, classified according to the EASL-CLIF definition of ACLF and acute decompensation. As already mentioned, the few available

studies are quite different, and only one a GWAS. Two of them conducted targeted genotype-phenotype association analysis, assessing specific polymorphisms that are thought to be involved in the host systemic inflammatory response [92,93]. Other studies included patients with HBV-related ACLF, that is paradigmatic of ACLF episodes in Asia [94–96].

Our GWAS, indeed, did not confirm previously reported results. Moreover, the identified SNPs, that show a significant association with patient phenotypes, were all located in non-codifying regions, thus not determining mutations directly affecting the functionality of codified proteins. At the same time, the SNPs identified in our study all belong to genetic loci that have been defined Cis-Regulatory Elements (CREs), since they may affect gene expression in multiple ways (some of them not completely understood). However, also this negative result deserves some comments, as well as a further insight on differences with previous studies.

First, our cohort of acutely decompensated patients was highly heterogeneous, compared to patients included in the Asian studies. ACLF diagnostic criteria according to the APASL definition [47] identify a peculiar clinical phenotype, characterized by an early stage liver disease (chronic hepatitis or compensated cirrhosis), a specific hepatotropic insult (mainly viral) and a primary liver failure without extra-hepatic involvement. We already dealt with the subject extensively, but it is worth remembering the main differences with ACLF according to the EASL-CLIF definition [21]. Indeed, many different insults (with a predominant role of bacterial infections) and many possible failing organs confer to the western phenotype of ACLF a great clinical heterogeneity. This observation allows highlighting a first limitation of our study, related to the sample size. Our cohort was comparable or superior to most available studies, in terms of absolute number of subjects, but at the same time did not allow to precisely discriminate many patient subgroups, because of the risk of an excessive granularity. We then decided to combine patients with infections and ACLF, based on a clinical and pathophysiological rationale, but there is no denying that both bacterial infections and ACLF present several very different phenotypes.

Since the genetic variants identified in previous studies were all related to components of the immune system, a second aspect that deserves to be considered is related to the underlying

immunological mechanisms in response to different infectious agents [110,111]. In cohorts of patients with HBV-related liver disease, host defense mechanisms have to face a single virus with common characteristics in all individuals. Conversely, in populations with ACLF triggered by many different bacterial infections, host immune system has to face highly heterogeneous pathogens with different mechanisms of action. Moreover, on the one hand, HBV-related chronic liver disease is a peculiar pathophysiological setting that involves immune tolerance and persistence of the viral agent [97]; on the other, significant genetic variants related to susceptibility of bacterial infections (e.g., primary immunodeficiencies) cause a high rate of infant or youth mortality [112].

Third, and further limitation of our study, is the absence of a validation cohort. Our findings were not compared to and validated on groups of patients with stable compensated liver cirrhosis (to define any genetic factor specifically associated with acute decompensating events) or healthy subjects (to exclude variants not specific for liver disease or cirrhosis).

In conclusion, some interesting findings can be pointed out and may represent the starting point for further research in this field. Our study, despite the above-mentioned limitations, substantially confirmed the scarce and weak evidence available so far regarding a genetic substrate underlying acutely decompensated cirrhosis. Therefore, it is reasonable to hypothesize that in adult patients with cirrhosis, acquired and environmental factors weigh more than genotype in affecting the clinical phenotype and determining short- and long-term outcomes. Moreover, the location of the identified SNPs within cCREs with regulatory effects on nearby genes can lead us to speculate on their potential effects in ACLF pathophysiology. FUT9 plays a role in CD15 expression on macrophages and mediates neutrophil adhesion to dendritic cells [113,114]. UFL1 contributes in regulating inflammatory response secondary to endoplasmic reticulum stress [115–117]. LMO7 is involved in Class I MHC mediated antigen processing and presentation, a fundamental process of the innate immune system involving Natural Killer cells. ACOD1 is involved in the modulation of TLR-mediated inflammatory response [118,119]. Although the above-mentioned methodological limitations, these findings may represent the basis for further research on this still open issue.

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