Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari

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CORSO DI DOTTORATO DI RICERCA IN: Scienze Mediche Cliniche e Sperimentali
CURRICOLO: Epatologia e Chirurgia Epatobiliare e Trapiantologica
CICLO 29°

# CHARACTERIZATION OF MOLECULAR PATHWAYS INVOLVED IN ACUTE AND ACUTE-ON-CHRONIC LIVER DISEASE

Tesi redatta con il contributo finanziario della Borsa Legge 170

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## Abstract (Italian)

**Introduzione:** Lo scompenso acuto in cirrosi è definito come la progressione acuta di una o più gravi complicanze della patologia epatica ed è la principale causa di ospedalizzazione nei pazienti con cirrosi. L'insufficienza epatica acuta su cronica (ACLF) è caratterizzata da uno scompenso acuto della cirrosi, da danno d'organo e da un elevato tasso di mortalità a 28 giorni. L'ACLF è caratterizzato da infiammazione sistemica e l'outcome infausto è strettamente associato con l'eccessiva risposta infiammatoria che si attiva nel paziente. L'inflammasoma è un complesso multi-proteico che attiva, mediante taglio proteolitico, citochine pro-infiammatorie come IL-1β e IL-18. Queste, hanno un ruolo nello sviluppo della patologia epatica. Le vescicole extracellulari (EVs) sono coinvolte in molti processi biologici importanti, sia fisiologici che patologici. Il processo di secrezione delle EVs da diversi tipi di cellule e la loro azione nel modulare l'avanzamento della patogenesi nella malattia epatica, non sono ancora completamente chiariti.

**Scopo:** Lo scopo di questo studio è caratterizzare la via del segnale coinvolta nello scompenso acuto della cirrosi e dell'insufficienza epatica acuta-su-cronica attraverso: la caratterizzazione del profilo infiammatorio dei pazienti arruolati, lo studio *in vitro* dell'effetto citotossico nel plasma dei pazienti nelle cellule tubulari renali (RTC), lo studio dell'espressione dell'inflammasoma nelle cellule trattate e nelle cellule mononucleate del sangue periferico (PBMC) estratte dai pazienti, la caratterizzazione delle EVs estratte dal plasma dei pazienti arruolati e lo studio del loro effetto *in vitro* su colture di RTC.

**Materiali e Metodi:** sono stati arruolati pazienti con cirrosi compensata, scompenso acuto in cirrosi, insufficienza epatica acuta-su-cronica e una popolazione di volontari

sani come controllo. I livelli plasmatici di IL-6, IL-1β e IL-18 sono stati misurati con kit ELISA. L'effetto citotossico del plasma nelle RTC è stato testato con il kit costituito da annexina V e propidio ioduro. Il livello di espressione delle molecole dell'inflammasoma è stato determinato sia nelle cellule stimolate con il plasma, sia nei PBMC estratti dai pazienti, attraverso Real Time Poly Chain Reaction (RT-PCR). Le EVs plasmatiche sono state estratte mediante ultracentrifugazione e la loro concentrazione determinata con il Nanosight. La loro caratterizzazione è stata eseguita mediante analisi al FACS. L'effetto citotossico delle EVs sulle RTC è stato determinato mediante saggio XTT.

**Risultati:** I livelli plasmatici delle citochine pro-infiammatorie, misurati nei primi pazienti arruolati, non mostra differenze tra i gruppi di pazienti con cirrosi compensata, scompensata e insufficienza epatica acuta-su-cronica. Anche la vitalità e la morte cellulare nelle colture stimolate con i plasma dei pazienti arruolati non hanno mostrato un profilo peculiare dei gruppi testati e non è stata riscontrata attivazione della trascrizione delle molecole dell'inflammasoma. L'espressione del Tool-like receptor 2 (TLR-2) nei PBMC si è dimostrata significativamente elevata nei pazienti con cirrosi compensata rispetto a quelli con scompenso acuto (p=0,036). Aggiungendo albumina al mezzo di coltura cellulare si è notata una riduzione dell'effetto citotossico del plasma dei pazienti nelle RTC. La concentrazione plasmatica di EVs risulta maggiore nei pazienti con insufficienza epatica acuta-su-cronica rispetto ai controlli sani. Le vescicole non esprimono i marcatori tipici piastrinici (CD41 e CD42b) e monocitari (CD14) sulla loro superficie ma esprimono il marcatore dell'epitelio attivato da piastrine (CD62E). I livelli di CD62E sono significativamente elevati nei pazienti con insufficienza eparica acuta-su-cronica rispetto ai controlli sani e ai pazienti con cirrosi

compensata (p=0,0041 e p=0,0111, rispettivamente). I livelli di CD40L sono significativamente elevati in tutti i gruppi di pazienti rispetto ai controlli sani (p<0,02). Le EVs isolate dei pazienti con insufficienza epatica acuta-su-cronica hanno un effetto citotossico superiore rispetto a quelle dei controlli sani e dei pazienti con cirrosi compensata nelle RTC (p<0,0001). Le cellule incubate con le EVs da pazienti con scompenso acuto in cirrosi e insufficienza epatica acuta-su-cronica vanno incontro ad apoptosi (p<0,0001), a produzione massiccia di ROS (p<0,0001), alla perdita della capacità di internalizzare l'albumina (p<0,0001) e alla riduzione dell'espressione di Zonula Occludens-1 (ZO-1) (p=0,0166) rispetto ai soggetti sani e ai pazienti con cirrosi compensata. Non si osserva invece un cambiamento nell'espressione cellulare di megalina e PGC1α.

**Conclusioni:** Il ruolo delle EVs nella cirrosi scompensata necessita di essere approfondito perché potrebbero rappresentare il veicolo su cui viaggiano i mediatori del danno d'organo extraepatico.

## ABSTRACT (English)

**Introduction:** Acute decompensation was defined as the acute development of one or more major complications of liver disease and it was the main cause of hospitalization in patients with cirrhosis. The acute-on-chronic liver failure (ACLF) was characterized by acute decompensation of cirrhosis, organ failure and high 28-day mortality. ACLF displayed key features of systemic inflammation and its poor outcome was closely associated with exacerbated systemic inflammatory responses. Inflammasomes were multiprotein complexes which proteolytically activates the cytokines IL-1β and IL-18. These substrates might have an effect on the development of liver disease. Extracellular vesicles (EVs) were involved in many important biological processes as well as in disease pathogenesis. The dynamics of EVs secretion by different cell types and how the secreted EVs interact to advance the pathogenesis of liver disease were still unknown.

**Aims:** The aim of this study was to characterize the molecular pathways involved in acute decompensation of cirrhosis and ACLF through: the characterization of the inflammatory profile of patients, the *in vitro* evaluation of cytotoxic effects of plasma from patients on renal tubular cells, the expression of Inflammasome in these treated cells and in Peripheral Blood Mononuclear Cells (PBMC) of patients, the characterization of EVs from patients and the study of *in vitro* effects of isolated EVs in renal tubular cells.

**Material and Methods:** We enrolled patients with compensated cirrhosis, acute decompensation in cirrhosis, ACLF and healthy subjects as control population. Plasma levels of IL-6, IL-1β and IL-18 were detected by ELISA assay. Cytotoxic effects of plasma on renal tubular cells were assayed by annexin V and propidium iodide kit.

Inflammasomes expression was detected both in renal tubular cells treated and in PBMC extracted from patients by Real Time PCR. Plasma EVs were extracted by ultracentrifugation and concentration was measured by Nanosight. Characterization of EVs was performed by FACS analysis. Cytotoxic effects of plasma EVs on renal tubular cells were assayed by XTT assay.

**Results:** Plasma levels of pro-inflammatory cytokines measure in the firsts patients enrolled did not differed between the groups of compensated cirrhosis, acute decompensation and ACLF. Also viability and death rate did not change in a way statistically significant in cell stimulated with plasma from the three groups of patients. Furthermore, Inflammasome gene expression in these cells did not underlines the activation of this protein complex. In PBMC from patients, gene expression of Tool-like receptor 2 (TLR-2) was significantly higher in patients with compensated cirrhosis compare to acute decompensation of cirrhosis ($p=0.036$). Albumin added to cell medium reduced cytotoxic effects of plasma on renal tubular cells. Plasma EVs of patients enrolled were more concentrated in ACLF groups compare to healthy subjects. EVs did not expressed selected platelets (CD41, CD42b) and monocyte markers (CD14) in their surface but they expressed marker of platelets activated endothelium (CD62E). The levels of CD62E were significantly higher in patients with ACLF compare to healthy subjects and patients with compensated cirrhosis ($p=0.0041$ and $p=0.0111$, respectively). CD40L levels were significantly higher in all patients' groups compare to healthy subjects ($p<0.02$). Plasma EVs from patients with acute and acute-on-chronic liver failure exerted a higher cytotoxic effects compare to healthy subjects and patients with compensated cirrhosis on renal tubular cells ($p<0.0001$). Cells incubated with EVs from acute and acute-on-chronic liver failure underwent to apoptosis ($p<0.0001$), to

ROS production ($p<0.0001$), to lose albumin intake capabilities ($p<0.0001$) and reduction of Zonula Occludens-1 (ZO-1) expression ($p=0.0166$) compare to healthy subjects and patients with compensated cirrhosis. Instead, megalin and PGC1α expression did not change.

**Conclusions:** The role of EVs in decompensated cirrhosis and ACLF need to be invastigated and study their hypothetic role as vehicle of mediator of extrahepatic organ injury and complications of cirrhosis.

# Introduction

## 1 Inflammatory response in Acute decompensation and Acute-on-chronic liver failure

Liver cirrhosis is the most advanced stage of chronic liver disease and represents a leading cause of mortality in adults, resulting in 1.03 million deaths per year worldwide (1). For several years cirrhosis has been considered a progressive disease characterized by a compensated phase followed by a decompensated phase and resulting invariable in death. In the last 20 years, with the development of effective etiological treatments, this concept has been overcome and cirrhosis has been considered a dynamic disease across compensated and decompensated stages (1). More recently, with the discovery of acute-on-chronic liver failure (ACLF) these concepts have been further revised. Indeed, it has been shown that in any stages of the disease an acute decompensating event may occur, leading to organ failure and conferring a high mortality risk (2).

### Definitions and outcomes

Acute decompensation is defined as the acute development of one or more major complications of liver disease (ie, ascites, encephalopathy, gastrointestinal hemorrhage, bacterial infection), and it is the main cause of hospitalization in patients with cirrhosis (3).

According to the European Association for the Study of the Liver Chronic Liver Failure (EASL-CLIF) consortium ACLF is characterized by 3 main features: presence of acute decompensation, organ failure (predefined by the SOFA-CLIF score) and high 28-day mortality rate (threshold of 15%) (4).

The Sequential Organ Failure Assessment (SOFA) scale which is used to diagnose organ dysfunctions and failures in general intensive care units (ICUs) (5). The SOFA score was found to be a better predictor of short-term prognosis of patients with cirrhosis than were liver-specific scores (the Child-Pugh and MELD scores). The investigators of the CANONIC study (4) used a modified SOFA scale, called CLIF-SOFA (**Table 1**). The CLIF-SOFA scale assessed the function of six organ-systems (liver, kidneys, brain, coagulation, circulation, and respiration) but also included some markers specific for cirrhosis. The definitions for organ failures based on the CLIF-SOFA scale were as follows: 1) Liver failure was defined by serum bilirubin levels of 12.0 mg/dL or more; 2) Kidney failure was defined by serum creatinine levels of 2.0 mg/dL or more, or the use of renal-replacement therapy; 3) Cerebral failure was defined by grade III or IV hepatic encephalopathy according to the West Haven classification; 4) Coagulation failure was defined by an International Normalized Ratio (INR) of more than 2.5 and/or platelet count of $20\times10^9$/L or less; 5) Circulatory failure was defined by the use of vasopressin (including terlipressin) to maintain arterial pressure; 6) Respiratory failure was defined by a ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (FiO2) of 200 or less or a pulse oximetry saturation (SpO2) to FiO2 ratio of 200 or less (**Table 1**).

ACLF has been graded in 3 stages:

Stage 1: Patients with serum creatinine (sCr) ≥ 2 mg/dl; patients with single failure of the liver, coagulation, circulation, or respiration who had a sCr level ranging from 1.5 to 1.9 mg/dL and/or mild to moderate hepatic encephalopathy (grade 1 or 2 according to West Haven criteria); and patients with single cerebral failure who had a serum creatinine level ranging from 1.5 and 1.9 mg/dL;

Stage 2: Patients with 2 organ failures;

Stage 3: Patients with 3 organ failures or more.

About 31% of patients with an acute decompensation of cirrhosis develop ACLF during the hospitalization. The occurrence of ACLF confers high risk of mortality, being 33% and 51% at 28-days and 90 days, respectively (4). Conversely, the prognosis of patients with an acute decompensation of cirrhosis without ACLF are quite better, being 2% and 10% at 28-days and 90-days, respectively. Finally, a stepwise increase in mortality rate has been found according to ACLF staging (23% for stage 1, 31% for stage 2 and 75% for stage 3 at 28 days).

**Table 1**: The Chronic Liver Failure (CLIF)-Sequential Organ Failure assessment (SOFA) Scale*
*(Moreau R., Clin Mol Hepatol. 2016; 22(1): 1–6)*

| Organ/system | Score | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Liver; Bilirubin, mg/dL | <1.2 | ≥1.2 - <2.0 | ≥2.0 - <6.0 | ≥6.0 - <12.0 | ≥12.0 |
| Kidney; Creatinine, mg/dL | <1.2 | ≥1.2 - <2.0 | ≥2.0 - <3.5 | ≥3.5 - <5.0 | ≥5.0 |
| | | | or use of renal-replacement therapy | | |
| Cerebral; HE grade† | No HE | I | II | III | IV |
| Coagulation; INR‡ | <1.1 | ≥1.1 - <1.25 | ≥1.25 - <1.5 | ≥1.5 - <2.5 | ≥2.5 or Platelets ≥20×109/L |
| Circulation; MAP mmHg | ≥70 | <70 | Dopamine ≤5 or Dobutamine or Terlipressin§ | Dopamine >5 or E ≤0.1 or NE ≤0.1 | Dopamine >15 or E > 0.1 or NE > 0.1 |
| Lungs; PaO2/FiO2: or | >400 | >300 - ≤400 | >200 - ≤300 | >100 - ≤200 | ≤100 |
| SpO2/FiO2Π | >512 | >357 - ≤512 | >214 - ≤357 | >89 - ≤214 | ≤89 |

HE denotes hepatic encephalopathy; INR, International Normalized Ratio; MAP, mean arterial pressure; E, epinephrine; NE, norepinephrine; PaO2, partial pressure of arterial oxygen; FIO2, fraction of inspired oxygen; SpO2, pulse oximetry saturation.
*Adapted from ref. 1. The highlighted area in violet depicts the diagnostic criteria for organ failures.
†The CLIF-SOFA scale used West Have classification while the original SOFA scale used the Coma Glasgow score.
‡INR was not included in the original SOFA scale.
§Terlipressin use was not taken into account in the original SOFA scale; doses for E and NE are expressed in µg/kg.min.
ΠThe SpO2/FiO2 ratio was not included in the original SOFA scale.

Multiorgan dysfunction and failure likely result from a complex interplay where the systemic spread of bacterial products represents the primary event. The consequent activation of the host innate immune response triggers endothelial molecular mechanisms responsible for arterial vasodilation, and also jeopardizes organ integrity with a storm of pro-inflammatory cytokines and reactive oxygen and nitrogen species. Thus, the picture of advanced cirrhosis could be seen as the result of an inflammatory syndrome in contradiction with a simple hemodynamic disturbance (6).

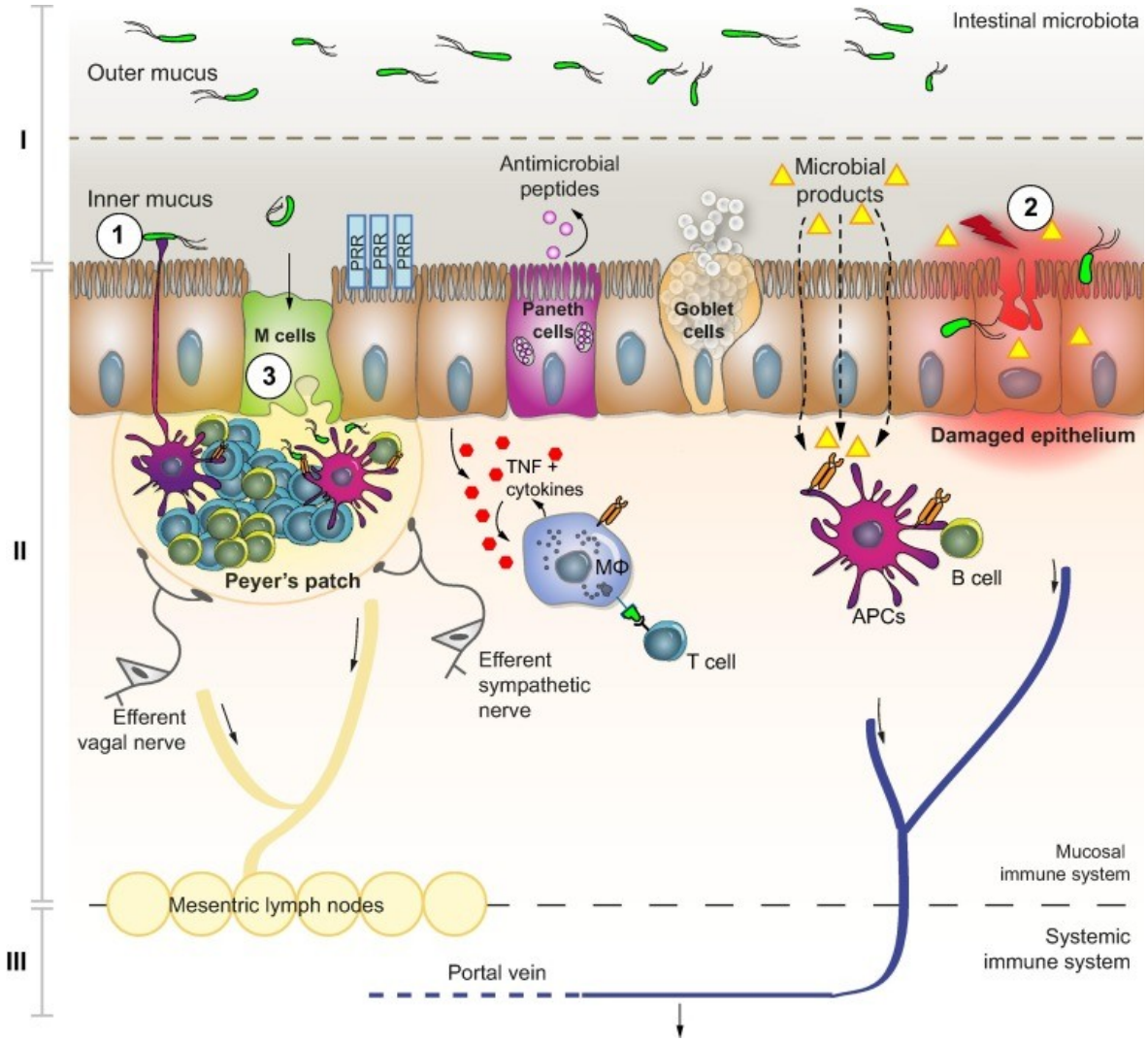
## Pathological bacterial translocation

Bacterial translocation was defined as translocation of bacteria and/or bacterial products (lipopolysaccharides, peptidoglycans, muramyl-dipeptides, bacterial DNA, etc.) from the gut to mesenteric lymph nodes (7). It was a physiological process in healthy conditions and crucial for host immunity. In contrast, in cirrhosis, ‘‘pathological’’ bacterial translocation developed with a sustained increase in quantity (rate and/or degree) of translocated bacteria (8).

The most evidenced clinical expression of pathological bacterial translocation was spontaneous bacterial peritonitis (SBP). SBP often originates from bacteria in the gut that belong to the normal intestinal microbiota. *In vivo* experiments showed that *E. coli* administered orally to cirrhotic rats was found not only in the intestinal lumen but also in the mesenteric lymph nodes and ascites (9). Furthermore, not only bacteremia but also inflow of translocating bacterial products into the hepato-splancnic and systemic circulation impacted on the cirrhotic host (10). Three were the main routes of bacterial translocation: (1) direct sampling of luminal bacteria by dendritic cells via processes between epithelial cells, not affecting tight junction function; (2) injured/inflamed

epithelium with dysfunctional epithelial barrer; (3) M-cells overlaying Peyer Patches as specialized cells providing access of microbial products to antigen-presenting cells (**Figure 1**).

**Figure 1**: Compartments and key players involved in mediating pathological bacterial translocation and the associated host response (Wiest R et al., *J Hepatol. 2014;60(1):197-209*).




One more time, three different levels of barrier against bacterial translocation were known (I-III, **Figure 1**): (I) lumen and secretory component (mucus layer, antimicrobial peptides) of gut barrier; (II) mechanical epithelial barrier and the gut-assiciated lymphatic tissue (GALT) beneath with response elements to bacterial translocation (pro-inflammatory cytokines) and autonomic nervous system; (III) systemic immune

system in case of spreasing of bacterial beyond mesenteric lymph nodes including hematogenous (portal venous) and lymphatic (ductus thoracicus) route of delivery (11). Rate and degree of pathological bacterial translocation increased with severity of liver disease: whereas they were increased in early cirrhosis, pathological translocation of viable bacteria occurs in the decompensated stage. Influencing factors that impact on the compartments driving pathological bacterial translocation were multiple and key players were pro-inflammatory cytokines, malnutrition (12), sympsthetic hyperactivity (13), genetic susceptibility (14, 15) and lack of bile acids (16, 17). After a priming events of paracellular translocation of bacterial products in early stage of cirrhosis, the main hypothesis was that epithelial tolerance, that normally avoids overwhelming mucosal inflammation, enhanced transcytosis of viable bacteria leading to immune paralysis in the GALT (18, 19). Augmented pro-inflammatory response to gut-derived products and failure to control invading bacteria and -products in concert with host susceptibility determined remote organ injury. This may included acute-on-chronic liver failure, hepato-renal-syndrome and hepatic encephalopathy (10). Therefore, understanding the physiology of gut-bacteria interactions and the pathogenesis of bacterial translocation can lead to new therapeutic targets in the prevention of infections and other complications of cirrhosis.

## Pathogenesis

The pathogenesis of acute-on-chronic liver failure is still debated, however, the common denominator seems to be the systemic inflammation (4). Indeed, white cell count and plasma C-reactive protein levels were found to be higher in patients with acute-on-chronic liver failure than those with acute decompensation. Similar findings

were found for plasma levels of several cytokines (for example: IL-6, TNF-α) and chemokines (IL-8) (20). Therefore, acute-on-chronic liver failure is characterized by an excessive inflammatory response during the "early ACLF" (2).

The inflammatory response can be triggered from so-called inducers, that can be exogenous (microbial and nonmicrobial) and endogenous, such as signals released from necrotic cells or products of extracellular matrix breakdown (21).

## Exogenous inducers

Bacterial infections have been considered to be a relevant trigger of ACLF. Indeed, almost 30% of cases of ACLF a bacterial infection can be identified as a potential precipitating event. Bacterial inducers are pathogen-associated molecular patterns (PAMPs) and virulence factors (21). PAMPS are recognize by pattern-recognition receptor (PRRs), which are receptors of host innate immune system and include toll-like receptors (TLRs), nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), retinoic acid-inducible gene- (RIG-), cytosolic DNA sensors. PRRs may be localized in cell surface or in the endolysosome compartment such as TLRs, or they may be cytosolic receptors such as NLRs, RIG-I and DNA sensors. When PAMPs bind PRRs, they trigger a signaling cascades that activate transcription factors. These last increase the transcription of antimicrobial effectors gene, cytokines and chemokines genes and other molecules of adaptive immunity (21, 22). When bacteria invade host cells, they may be recognized and bound by intracellular PRRs.

After binding of PAMPs, PRRs assemble into high-molecular weight, caspase-1-activating platforms called ‘‘inflammasomes’’ that control maturation and secretion of interleukins such as IL-1β and IL-18, whose potent pro-inflammatory activities direct host responses to infection and injury (23).

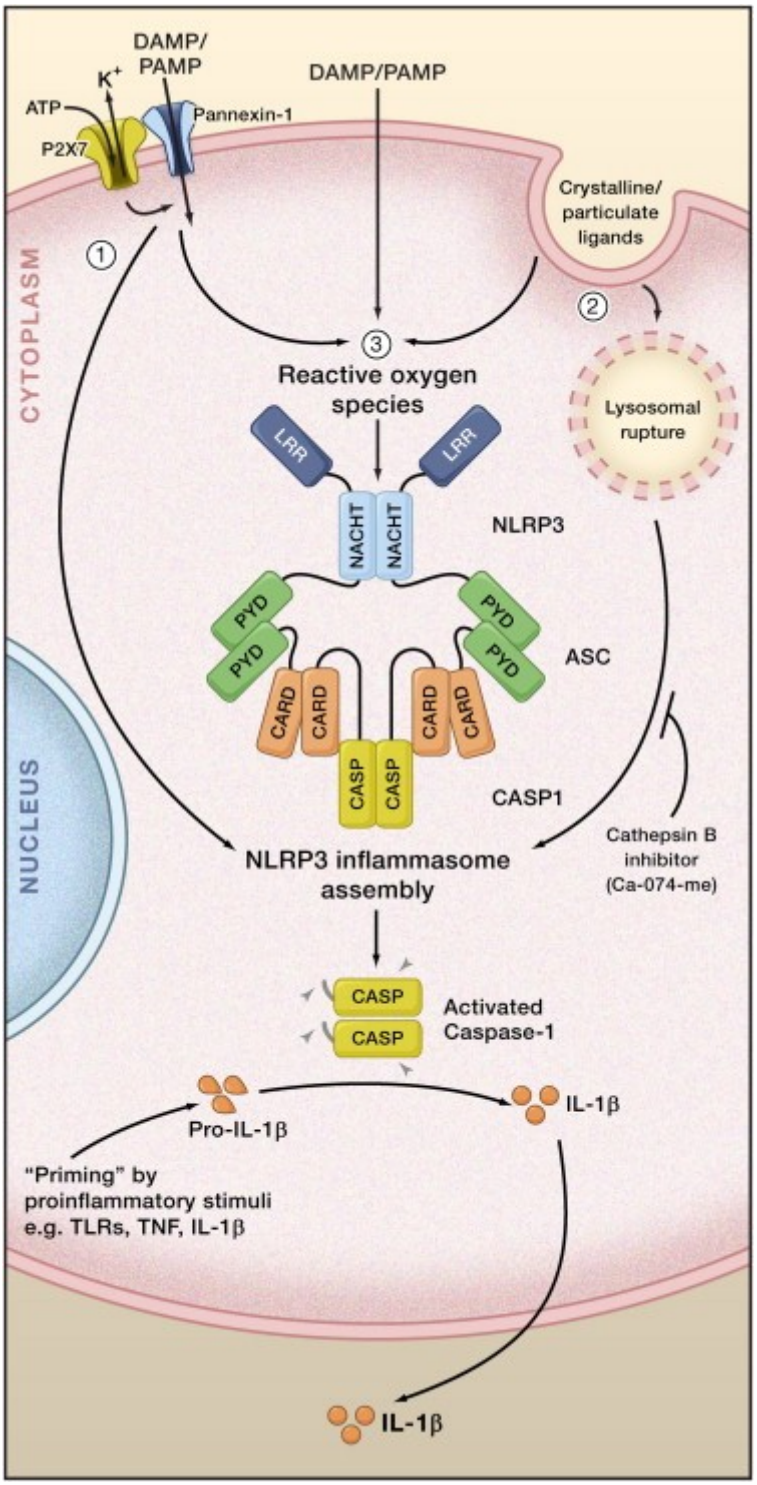
## Inflammasomes

Inflammasomes are platforms for Caspase-1 activation and IL-1β maturation (23). Caspases are cysteine proteases involved in inflammation and cell death. Their potent cellular activities are controlled by proteolytic activation. Caspase-1 is a pro-inflammatory caspase whose catalytic activity is tightly regulated by signal-dependent autoactivation whithin inflammasomes (24). IL-1β is a pro-inflammatory mediator generated in the site of injury and it is involved in cellular recruitment to a site of infection or injury. Pro-inflammatory stimuli induce expression of the inactive IL-1β proform, but cytokine maturation and release are controlled by inflammasomes.

Inflammasomes are assembled by self-oligomerizing scaffold proteins. NLR family member showed inflammasome activity *in vitro*. In fact, NLRP3 inflammasome is currently the most fully characterized inflammasome and consist of the NLRP3 scaffold, the ASC (PYCARD) adaptor, and Caspase-1 (23).

Upon NLRP3 activation, NLRP3 oligomerization leads to PYD domain clustering and presentation for homotypic interaction with the PYD- and CARD-containing the adaptor ASC, whose CARD domain in turn recruits the CARD of procaspase-1. Procaspase-1 clustering permits autocleavage and formation of Caspase-1, which then processes cytokine maturation and secretion of pro-inflammatory cytokines, such as interleukin-1β and IL-18 (23)(**Figure 2**).

**Figure 2**: NLRP3 Inflammasome Activation (*Schroder K. et al. Cell 2010 (140):821-832).*



### Endogenous inducers

Endogenous inducers are produced from tissue damage or injury. They are molecules released by necrotic cells and product of breakdown of the extracellular matrix. They are called danger-associated molecular patterns (DAMPs) and they are recognized by sensor expressed at the surface of resident macrophages and trigger inflammation (25). Necrosis can be result of the induction of programmed cell death, and in this case includes processes of pyroptosis and necroptosis. Only pyroptosis results from inflammasome activation (26).

Necrotic cells release adenosine triphosphate (ATP) that can be binded from PRRs and contribute to NLRP3 inflammasome activation in macrophages (22).

### Immunopatology and Outcomes

The aim of the inflammatory response to bacterial infection is to promote host resistance by reducing bacterial burden, instead the aim of the DAMPs-mediated response to tissue injury is to promote tissue repair (26). Both the two categories of inflammatory response, when excessive, may induce tissue damage. The excessive inflammatory response to bacterial infection that cause collateral tissue damage is a process called immunopathology. Bacterial are potential causes of immunopathology, but they can exert tissue damage also via toxins and virulence factors (21, 26). Severe outcome of bacterial infection can be caused by failure of protective tissue-intrinsic mechanisms. Several studies report that severity of the disease may be related to failed disease tolerance (27-29).

Immunopatology is a major mechanism explaining the development of ACLF in patients with SBP. SBP caused by gram-negative bacteria of intestinal origin is the most

common infection in patients with cirrhosis, and it is more common in patients with ALCF than in those without (4). In case of SBP, the greater the intensity of the immune response, the higher the risk of developing Hepatorenal Syndrome Type 1, a form of ACLF (30).

Immune response is in general dependent from age and from genetic factors. Patients with ACLF are younger than those without (4) and show a more vigorous immune response to infection (27). Regarding genetics factors, it was demonstrated that variants of cytosolic receptor NOD2 may contribute to susceptibility to intestinal bacterial translocation, in a manner NFkB dependent (15), and to the poor outcome of patients. Single-nucleotide polymorphism (SNPs) in gene encoding PRRs (TLR2, TLR4) or nuclear receptor are associated with an increased risk of bacterial infection in patients with cirrhosis (31). Recently, Alcaraz-Quiles J. et al. identify two common functional polymorphisms in the IL-1 gene cluster (rs1143623 in IL-1β gene and rs425196 in IL-1ra) which are associated with the inflammatory process related to the development of ACLF (32).

Almost 20% of the cases of ACLF are precipitated by alcohol consumption and in some cases may have the characteristic of alcoholic hepatitis (4). The main features of alcoholic hepatitis are hepatocyte death and liver inflammation, with prominent neutrophil infiltration (33).

Patients with excessive alcohol consumption have gut dysbiosis and increase of permeability of intestinal barrier (11, 33). As a consequence, translocation of bacterial PAMPs may occur. PAMPS were recognized and binded by TLRs in liver resident macrophages. This binding to TLRs stimulates the production of pro-inflammatory cytokines that are potent neutrophil-attracting cues. Therefore, on the one hand,

production of pro-inflammatory cytokines and mediators can lead to tissue damage, on the other hand there are findings suggesting that the primary purpose of inflammatory response might be to promote tissue repair/regeneration. In fact, it has been observed a better prognosis the higher is the liver infiltration by neutrophils (34). Activity of TNF-α, induced by lipopolysaccharide (LPS) of gram-negative bacteria, stimulate production of reactive oxygen species (ROS) from hepatocytes and so oxidative stress (33). Subsequently, ROS and LPS induce hepatocyte endoplasmatic reticulum stress, that together with oxidative stress trigger a cell-extrinsic response that contains homeostasis-restoring components (35). Patients with severe alcoholic hepatitis also develop extrahepatic organ failures but it is still unclear whether an excessive systemic inflammatory response can lead to extrahepatic organ failure.

At last, approximately 50% of cases of ACLF is of unknown origin, but probably they are associated with systemic inflammation (4). Three hypotheses have been proposed: the presence of gut dysbiosis, the role of translocation of bacterial PAMPs and the action of endogenous inducers of inflammation (as shown above).

In patients with gut dysbiosis, it was shown a decrease of *Bacteroidaceae* and an increase of *Enterobacteriaceae*, but this is dependent from stage of cirrhosis and its severity (36-40). However, changes in gut microbiome correlates with a more intense systemic inflammation, probably due to metabolites produced by intestinal microbiome itself (40).

About bacterial translocation, it was shown that bacteria could release PAMPs that reach systemic circulation, it can be recognized by TLRs at different sites and trigger

inflammation (41, 42). Generally, TLR recognition is not dependent on bacteria viability or invasiveness (26).

Among ACLF patients, indipendently from levels of systemic inflammation, patients who have prior episodes of decompensation of liver disease have a lower risk of death than those who do not have prior episodes of decompensation (4). It has been hypothesized that patients may be primed by prior episodes of decompensation and become more tolerant when exposed to new noxious stimuli.

An extreme inflammatory response is not the only feature of ACLF. Interestingly it has been shown that in patients with ACLF circulating CD14+ immune cells are enriched MER-expressing subset of cells which exhibit decreased responses to LPS stimulation. MER receptor have a role in the clearance of apoptotic cells by phagocytes and it is the expression product of MERTK gene. The induction of MERTK occurs in response to stimuli homeostatic-restoring signals (43). Therefore, CD14+ cells expressing MER receptor may be engaged to protect damage tissues in ACLF contest.

Further stressing these concepts it has been shown that plasma from patients with acute decompensation had increased levels of prostaglandin E2 that may inhibit the macrophages TNF-α production in response to LPS (44) and decrease the macrophages ability to kill bacteria.

This findings suggests that some patients with ACLF and acute decompensation of cirrhosis show an immuneparalysis that may confer a predisposition to the development of new infections, that impairs survival in patients with cirrhosis (45). In patients with ACLF it may represent a sort of compensatory anti inflammatory response that is a well known condition in patients with sepsis (46).

# 2 Organ crosstalk

The human organism is organized in various organ systems that are intimately connected to each other. The concept of “crosstalk” has not been clearly defined . The term comes from electronics, denoting any signal or circuit that unintentionally affects another. In molecular biology, crosstalk is used to describe instances when one or more components of signal transduction pathway affect a different pathway, either at the transmembrane or at the intracellular level. The “organ crosstalk” can be defined as the complex biological communication, signaling and feedback between distant organs mediated via cellular, molecular, neural, endocrine and paracrine factors. The physiological crosstalk is necessary to maintain regular homeostasis and normal functioning of the organism. Nevertheless, a pathological crosstalk can also develop when the effect of one malfunctioning and damage organ induces structural and/or functional dysfunction in distant organs. This effect is usually negative since it results injurious and destructive for the target organs and, as consequence, for the whole organism (47-50). Thus, in the diseased state, the crosstalk can trigger an organ disequilibrium and create a vicious damage in distant organs (49, 50).

## Liver and Kidney crosstalk

Liver and kidney are important regulators of body homeostasis and are involved in excreting the toxic byproducts of metabolism and exogenous drugs (51). The intersection of liver and kidney disorders is so frequent in clinical practice that it seemed likely the hypothesis of a bidirectional crosstalk. There are a number of potential contributing causes for liver-kidney crosstalk that may predispose to the development of this vicious interaction and which are relevant for the susceptibility,

etiology, severity and duration of the disease state. Severity of the failing organ can initiate various complex metabolic, cell mediated and humoral pathways affecting distant organs, contributing to the high therapeutic costs, and significantly higher morbidity and mortality. The severity of liver damage often parallels the severity of kidney injury and viceversa (51, 52). An acute kidney injury (AKI) is common in patients with a severe liver disease while AKI may per se induce a liver damage by promoting oxidative stress, inflammation and apoptosis.

IgA Nephropathy and Hepatorenal Syndrome are examples of pathological crosstalk. A high incidence of IgA nephropathy has been reported in patients with liver cirrhosis, though, clinically evident nephrotic syndrome is very uncommon. Impaired hepatic clearance of circulating IgA immune complexes and subsequent deposition in renal glomeruli has been considered principally in the pathogenesis of liver cirrhosis associated IgA nephropathy (53). Hepatorenal syndrome (HRS) is a functional form of acute kidney injury (AKI) that develops in patients with advanced cirrhosis or fulminant hepatic failure. It is the cause of deterioration of kidney function in only a fraction of all AKI cases diagnosed in cirrhotic patients (54).

The liver-kidney crosstalk in the disease state is poorly understood. A better understanding of this bidirectional crosstalk may offer improved management strategies but it encompasses several mechanisms of cell and tissue damage and/or dysfunction.

# 3 Extracellular vesicles

Several mechanisms involved in cell-to cell communication have been identified, including secretion of growth factors, cytokines, surface receptors, and nucleotides. Recent studies have suggested that cells may also communicate by membrane-derived nanometer-sized vesicles termed Extracellular Vesicles (EVs). For a long time, EVs were considered to be inert cellular debris and the frequently observed vesicles by electron microscopy in the interstitial space of tissues or in blood were considered the consequence of cell damage or the result of dynamic plasma membrane turnover. EVs are released by normal and diseased cells, both *in vitro* and *in vivo*. They have been identified in all major bodily fluid, including blood, urine, bile, saliva, semen, cerebrospinal fluid, as well as in cirrhosis-associated ascites (55). EVs are involved in many important biological processes as well as in disease pathogenesis (56, 57). EVs represent an heterogeneous population, differing in cellular origin, number, size, antigenic composition, and functional properties.

## Classification of EVs

Based on their cellular biogenesis, EVs are classified into three groups: exosomes, microvesicles and apoptotic bodies (58). Exosomes are EVs derived from intracellular trafficking via the endolysosomal pathway: they are intraluminal vesicles contained in multivesicular bodies (MVBs), which are released to the extracellular environment upon fusion of MVBs with the plasma membrane. The size of exosomes is estimated to range between 40 and 150 nm in diameter (59). Microvesicles are vesicles of different sizes (usually from 50 to 1000 nm in diameter) which bud directly from the plasma membrane. Lastly, apoptotic bodies, usually greater than 500 nm in diamater,

represent cell fragments and they are formed by large-scale plasma membrane blebbing during apoptosis.

## Communications via EVs

The most critical function of EVs is intercellular communication. This may be paracrine, endocrine, and also autocrine. Communication via EVs involves two facets: target cell recognition and signal transfer to the target cell (60).

EVs released from a given cell type may interact through specific receptor ligands with other cells, leading to target cell stimulation directly or by transferring surface receptors.

This implicates that MVs interact only with target cells that specifically recognize rather than just with any cell present in the microenvironment. This interaction may either be limited to a receptor-mediated binding to the surface of target cells forming a platform for assembly of multimolecular complexes or leading to cell signaling, either to be followed by internalization as a result of direct fusion or endocytic uptake by target cells.

Once internalized, MVs can fuse their membranes with those of endosomes, thus leading to a horizontal transfer of their content (mRNAs, microRNAs and proteins) in the cytosol of target cells. Alternatively, they may remain segregated within endosomes and be transferred to lysosomes or dismissed by the cells following the fusion with the plasma membrane, thus leading to a process of transcytosis.

Several studies have demonstrated that RNA, DNA, and proteins are encapsulated in EVs as bioactive molecules. These play important role in intercellular communication, and in specific signal transduction in the pathophysiological states of cells both *in vitro* and *in vivo* (61-63).

In patients suffering from inflammation evoked by various diseases, EVs are often observed in plasma and in other body fluids (64-67) and the number of EVs are increased in response to the disease severity. In particular, the number of EVs has been observed to increase in the bloodstream of patients suffering from acute and chronic inflammation evoked by diseases such as sepsis, stroke, preeclampsia, atherosclerosis, diabetes mellitus, metabolic syndrome, and cancer (65, 67-71). Moreover, Hergenreider E. and Yamamoto S. (63, 72) showed that cells under inflammatory conditions actively communicate with their adjacent cells through EVs.

## Biological activities of EVs

EVs exert their effects on fundamental biological processes in a pleiotropic manner, directly activating cell surface receptors via protein and bioactive lipid ligands, merging their membrane contents into the recipient cell plasma membrane and delivering effectors including transcription factors, oncogenes, small and large non-coding regulatory RNAs, mRNAs and infectious particles into recipient cells (73-75)(**Figure 3**).

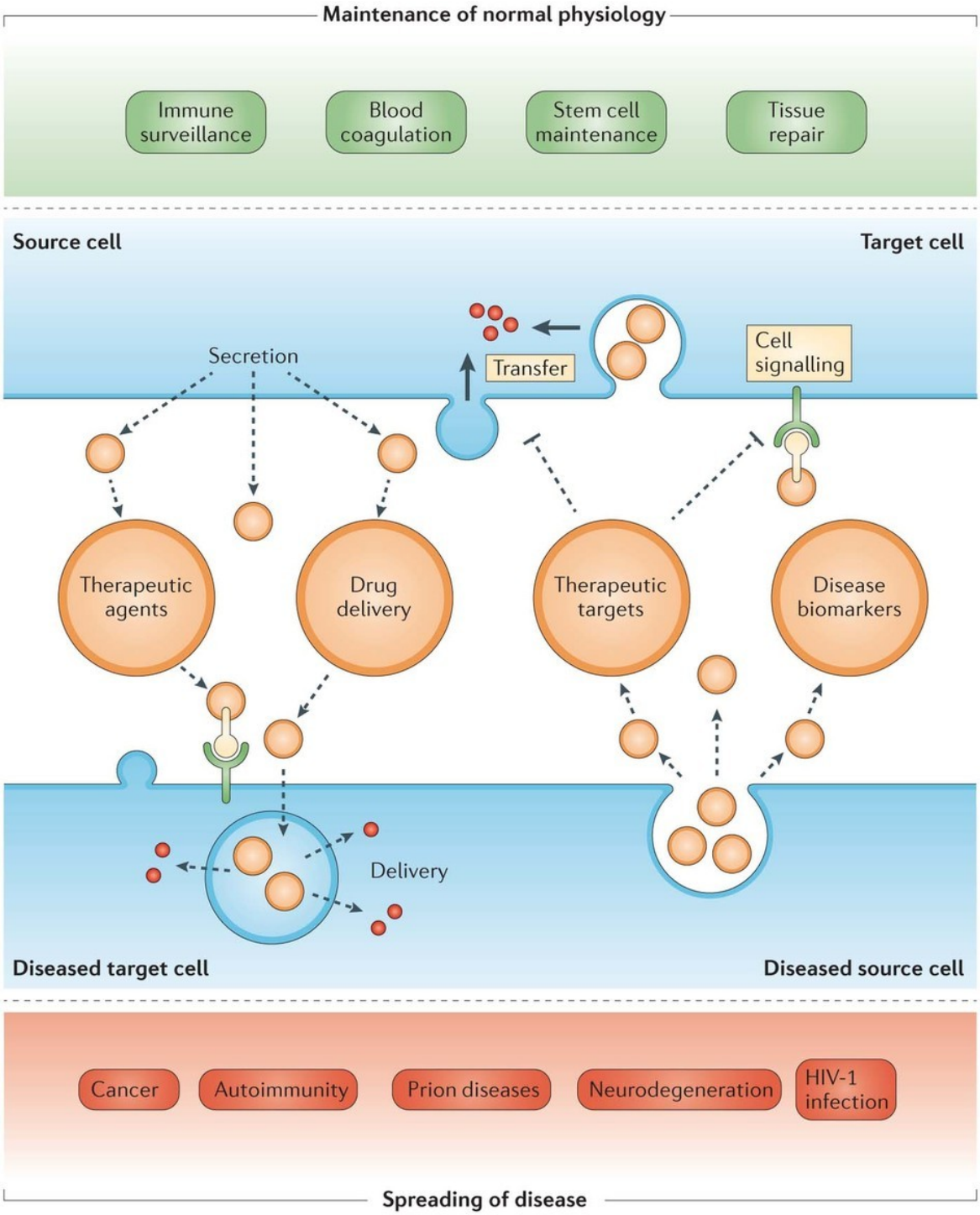
- Manteinence of normal physiology: stem cell mainteinance, tissue repair, immune sorveillance and blood coagulation (64, 76);
- Signalosomes: EVs can act as multifunctional signalling complexes for controlling fundamental cellular and biological functions (in the regulation of immune responses (77), in immune suppression (78) and immune activation (79);
- Cell phenotype modulation: for example, in converting the hematopoietic stem cell phenotype into a liver cell phenotype (80);

- Stem cell maintenance and plasticity: stem cell-derived EVs have a pivotal role in tissue regeneration following injury (81);

Pathological role of EVs

- Stimulation of tumour progression: EVs induce proliferation in cells, stimulating tumor grown, stimulating angiogenesis, promoting matrix remodeling and inducing metastasis (75, 82);
- Spread of pathogens: HIV-1, Epstein-Barr virus and prions (83-85);
- Local propagation of neurodegenerative disease: Alzheimer Disease (86) and Parkinson's Disease (87).

**Figure 3**: Role of extracellular vesicles in normal physiology and disease pathogenesis (*Nature Reviews Drug Discovery 12, 347-357 (2013)*)



## EVs in liver disease

As it happens for cardiovascular disorders, thrombosis and cancer, also in case of liver cirrhosis circulating levels of EVs are increased. Some studies described this phenomena as a result of increased formation and/or decreased clearance of EVs.

Causes which trigger EVs production are: alcohol consumption (88), viral infection (89), diabetes (90), obesity (91), dyslipidaemia (92) and physical inactivity (93). Processes in liver disease that increase EVs formation are apoptosis and cell activation. Moreover, stimuli for EVs release are oxidative stress (94), shear stress (95), systemic inflammation and bacterial translocation (96, 97).

Under healthy conditions, spleen and liver macrophages are the primary contributors to EVs clearance from the circulation (98). It is well known that cirrhosis is associated with a defect in macrophage function, so Lemoinne S. et al. (99) speculate that clearance of EVs might be decreased in these patients.

## EVs in liver disease progression

In physiological condition, extracellular vesicles are released at low levels from almost all cell types. Most of them are derived from platelets and endothelial cells (100) and have been shown to be important in haemostatic events such as coagulation. Nevertheless, the dynamics of EV secretion by different cell types and how the secreted EVs interact to advance the pathogenesis of a given disease is still unknown. Controlled *in vitro* study which involved liver injury model to explore EV-mediator fibrosis (101-103), transcriptomic signaling (104-108) and targeted immunotherapy (109-111) in artificial cell culture system. However, *in vivo* studies present an added degree of complexity due to the difficulty of identifying liver specific EVs within the

circulating pool. For this reason, several studies focus on EVs characterization and temporal changes in relation to liver disease development (112-114).

### Role of EVs in liver fibrogenesis, portal hypertension and activation of coagulation

In hepatic fibrosis the excessive extracellular matrix is produced by activated mesenchymal cells which resemble myofibroblasts. They derive from quiescent hepatic stellate cells (115). It was shown that the same cells that drive fibrogenesis (hepatic stellate cells) can become major effectors of fibrolysis via production and activation of certain matrix metalloproteinases (MMPs) (116). Kornek et al. (103) demonstrated that T cell EVs circulate in blood and are elevated in patients with active chronic hepatitis C compared with patients with mild hepatitis C and healthy controls. Further, EVs derived both from CD8+ and CD4+ T cells can induce a fibrolytic phenotype in hepatic stellate cells. This activity depends on fusion of the plasmatic membrane with hepatic stellate cells membranes and transfer of T cell membrane molecules such as CD147 to hepatic stellate cells in a partly CD54 (ICAM-1) dependent manner. Moreover, indirect evidence indicates that EVs could also promote fibrolysis via the microRNAs they countain (117).

Angiogenesis is proposed to promote fibrogenesis in the liver (118). Angiogenesis is increased by EVs generated *in vitro* from platelets, lymphocyte, and endothelial progenitors cells. Published results of Feldstein and colleagues (119) suggest that hepatocyte derived EVs contribute to angiogenesis and liver fibrosis in steatohepatitis, in a manner depandent on Vanin-1. In sight of this, the hypothesis is that the proangiogenic effect of EVs may have a role in promotion of fibrogenesis in the liver.

Portal hypertension is a frequent and severe complication of cirrhosis. It is a combination of structural and dynamic components. The structural components include fibrosis, regenerative nodule formation and vascular remodelling. EVs role in fibrosis is discussed above, but they might also contribute to vascular remodelling in cirrhosis through the release of EVs expressing Hedgehog ligands from hepatic stellate cells. These ligands induce the expression of inducible nitric oxide syntase (iNOS) (120). Moreover, portal myofibroblast release EVs that stimulate angiogenesis in a VEGF-dependent manner, enhancing vascular remodeling (121).

In cirrhotic patients, progressive splanchnic arterial vasodilatation may aggravates portal hypertension. EVs seem to contribute to vascular hypocontractility in patients with advance cirrhosis. This was demonstrated from Rautou et al. (122), which showed that patients with Child-Pugh B or C cirrhosis have increased circulating levels of EVs of Leukoendothelial, lymphocyte, erythrocyte and hepatocyte origin. These EVs decrease arterial blood pressure in mice through the transfer of phospholipids from EVs to endothelial cells. Interestingly, this was not observed with EVs from patients with Child-Pugh A cirrhosis.

### Role of EVs in hepatocellular carcinoma

A study, published from Brodsky at al. (114), in a small group of HCC patients reported association between tumor size and plasma levels of hepatic and endothelial EVS. Furthermore, number of EVs decrease after HCC removal by liver transplantation. Many other group reported investigation of the role of EVs in the development of cancer. First of all, EVs promote angiogenesis. EVs containing the oncogenic epidermal growth factor receptor (EGFR) that could be taken by endothelial cells. Second, EVs

containing active oncogene (64). Third, EVs might also favour multidrug resistance, a major cause of unsuccessful cancer treatment (123). In the end, EVs may facilitates tumor invasion and metastasis, stimulating expression of matrix metalloproteinase in fibroblast by CD147 transport from T cell (124).

### Circulating procoagulant EVs in patients with acute liver failure

Patients with acute liver failure seem to be prone to thrombosis than to bleeding complication, and intrahepatic thrombosis might exacerbate initial liver injury (125). The procoagulant activity of EVs, due to presence of phosphatidylserine and tissue factor at their surface, in patients with acute liver injury is fourfold and 38-fold higher respectively than that in healthy controls (126, 127).

### EVs as diagnostic and prognostic biomarkers in patients with liver disease

A great interest has recently been directed toward using EVs as biomarker for disease diagnosis and prognosis. EVs isolated from several biological fluids (urine, bile, serum) can be characterized with proteomic, genomic and lipidomic approaches.

Levels of EVs originating from CD4+ and CD8+ T cells are increased in patients with chronic hepatitis C, whereas levels of EVs from CD14+ and invariant natural killer T cells are augmented in patients with Non-alcoholic Fatty Liver Disease (NAFLD). This observation may be helpful in identifying the cause of liver blood test abnormalities in patients with HCV infection and the metabolic syndrome after liver transplantation (128).

In liver rejection, EVs may be helpful in discriminating case of acute rejection, because EVs levels progressively decrease after surgery in patients without rejection (114).

Circulating EVs are potential noninvasive markers of disease severity and activity in chronic hepatitis C, NAFLD and cirrhosis (103).

EVs levels were associated with the presence of the systemic inflammatory response syndrome. Multivariate logistic regression analysis showed that EVs concentration were indipendently associated with death and liver transplantation in patients with acute liver failure (127). Most of these circulating g EVs are CD41+. Thus, these originate from platelets, supporting the hypothesis that the severity of acute liver failure is related to systemic inflammatory response syndrome and coagulopathy.

### EVs and the complication of cirrhosis

Probably, EVs don't have a role in the complications of cirrhosis, except for initial phase of development of portal hypertension. However, several properties of EVs contribute to ascites, hepatic encephalopathy, hepatopulmonary syndrome, portopulmonary hypertension and hepatorenal syndrome.

The EVs ability to increase endothelial permeability and consequently, to contributed to the formation of ascites is still debated. Furthermore, EVs were found in the ascites of patients with ovarian and colorectal cancer but not in ascites of patients with cirrhosis (129).

Instead, EVs contribute to hepatic encephalopaphy for several reasons: patients with cirrhosis and encephalopathy have 3.5-fold more leukoendothelial EVs than those without encephalopathy (122); EVs might increase blood-brain barrier endothelial cell permeability (130); brain endothelial cells might release EVs that induce astrocyte swelling (131).

The role of EVs in hepatopulmonary syndrome has not yet been assessed, but potentially EVs can modulate the main mechanism (pulmonary vasodilation and angiogenesis) involved in the development of this syndrome.

Hepatorenal syndrome is characterized by functional renal failure in patients with cirrhosis and ascites. EVs may contributed to systemic and splancnic vasodilation (122). Moreover, EVs have been shown to induce renal vasoconstriction in sickle cell disease (132).

## AIMS of THE STUDY

The aim of this study was to characterize the molecular pathways involved in acute decompensation of cirrhosis and acute-on-chronic liver failure. This aim was pursued through the following intermediate goals:

- Characterization of the inflammatory profile of patients by detection of pro-inflammatory cytokines in plasma;

- Evaluation of cytotoxic effects of plasma from patients with compensated cirrhosis, acute decompensation and acute-on-chronic liver failure on renal tubular cells considering the role of organ crosstalk on pathophysiology of liver disease;

- Evaluation of expression of Inflammasome in renal tubular cells stimulated with plasma from patients with compensated cirrhosis, acute decompensation in cirrhosis and acute-on-chronic liver failure and in (PBMC) isolated from the same groups of patients;

- Characterization of EVs from plasma of patients with compensated cirrhosis, acute decompensation and acute-on-chronic liver failure and healthy subjects like a vehicle of communication between organs;

- Study of *in vitro* effects of EVs, extracted from plasma of patients with compensated cirrhosis, acute decompensation, acute-on-chronic liver failure and healthy subjects, in cells of renal tubular epithelium in order to investigate their role on pathogenesis of ACLF.

# Material and Methods

## Patients

### Inclusion and Exclusion Criteria

Outpatients with compensated cirrhosis: Inclusion criteria were as follows: (1) cirrhosis as diagnosed by liver biopsy or clinical, biochemical, ultrasound, and/or endoscopic findings; (2) age >18 years. Exclusion criteria were the follows: (1) hepatocellular carcinoma beyond Milan criteria; (2) ascites; (3) positive urine culture; (4) therapy with albumin; (5) autoimmune diseases; (6) genetic emocromatosis; (7) varices; (9) previous stories of decompensation.

Patients with acute decompensation in cirrhosis: Inclusion criteria were as follows: (1) cirrhosis as diagnosed by liver biopsy or clinical, biochemical, ultrasound, and/or endoscopic findings; (2) age >18 years; (3) one or more major complications of liver disease (ie, ascites, encephalopathy, gastrointestinal hemorrhage, bacterial infection). Exclusion criteria: (1) liver transplantation; (2) Hepatocellular carcinoma; (3) hospitalization for scheduled procedures.

ACLF patients: Inclusion criteria: (1) cirrhosis as diagnosed by liver biopsy or clinical, biochemical, ultrasound, and/or endoscopic findings; (2) age >18 years; (3) ACLF diagnosed according to the the EASL-CLIF consortium definition (Moreau et al Gastroenterology 2013).

### Study design and samples collection

Demographic, clinical and routine laboratory data were collected at the inclusion in the study. Peripheral venous blood samples were collected from patients with Acute Decompensation and Acute-on-Chronic Liver Failure at diagnosis; patients with

compensated cirrhosis were enrolled at outpatients visit. Blood samples were collected in EDTA tubes and subsequently centrifuged for 10 min at 3,500 rpm. Following centrifugation, plasma was immediately separated from blood cells and stored at −80°C until use. All samples were processed within 4 h after collection. Collection and processing of control samples from healthy volunteers followed an identical protocol.

### Determination of IL-6, IL-1β and IL-18 in plasma

Plasma was assayed for IL-6, IL-1β and IL-18. Cytokines concentration was measured by human instant enzyme-linked immuno-sorbent assay (ELISA) kit (eBioscience, San Diego, Calif., USA) with a fluorometric assay. Plasma was processed according to the manufacturer's instructions. Cytokines levels were measured in plasma at 450 nm by VICTOR X4 multilabel plate reader (Perkin Elmer Life Sciences, Waltham, Mass., USA) and concentrations (pg/ml) were calculated from the standard curve according to the manufacturer's protocol. Standard samples ranged from 7.8 to 500 pg/ml for IL-1β, from 3.1 to 200 pg/ml for IL-6 and from 78 to 5000 pg/ml for IL-18. Human IL-1β instant ELISA kit sensitivity is 0.7 pg/ml, human IL-6 instant ELISA kit sensitivity is 0.92 pg/ml, and human IL-18 instant ELISA kit sensitivity is 9.2 pg/ml.

### Isolation of PBMCs from blood

Human PBMC were isolated from 10 mL of venous blood using Ficoll–Hystopaque density centrifugation. After the centrifugation at 2200 rpm for 20 minutes at room temperature without brake, PBMC were collected from the interphase layer and washed with physiological solution (0,9% p/V NaCl)(Monico SPA, Mestre Venezia). PBMC were suspended in RPMI 1640 supplemented with 40% (v/v) FBS and storage at -80°C until further processing.

## RTC Culture

Primary cultures of human proximal Renal Tubular Cells (RTCs) were obtained from kidneys removed by surgical procedures from patients affected by renal carcinomas. An immortalized human proximal RTC line was generated by infection with a hybrid Adeno5/SV40 virus. The purity of the primary cultures was assessed on the basis of cell characterization, according to published criteria (133). RTCs were cultured in completed liquid-phase medium (RPMI-1640, PBI International, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum for 30 min at 56°C, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Sigma Chemical Co., St. Louis, Mo., USA). These cells were maintained in a controlled atmosphere (5% $CO_2$) at 37°C and passaged at 80% confluence checked by an inverted microscope.

## Induction of Apoptosis

RTCs were treated with participants' plasma from the Compensated Cirrhosis, Acute Decompensation and healthy subject groups. The ability of plasma to induce apoptosis was evaluated at 24 h. Untreated cells were maintained in the same manner and used as an internal control.

RTCs were plated at $2 \times 10^6$ cells per well in 6-well plates and incubated with 90% RPMI-1640 medium (with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin) and 10% of EDTA plasma from the Compensated Cirrhosis, Acute Decompensation and healthy subject groups in the standard condition (at 37°C in 5% $CO_2$ for 24 h). Prior to use, RTCs were washed twice in Dulbecco's phosphate-buffered saline (PBS; without calcium and magnesium), pH 7.4. Each incubation was performed in triplicate.

## Detection of cell viability by flow cytometry

The Annexin V-FITC kit (Beckman Coulter, Brea, Calif., USA) is an apoptotic detection kit based on the binding properties of annexin V to phosphatidylserine and on the DNA-intercalating capabilities of propidium iodide (PI). Cells were washed twice with cold Dulbecco's PBS and resuspended in 500 µl of PBS at a concentration of 1 × $10^6$ cells/ml. 100 µl of this solution was incubated by 5 µl of FITC-conjugated annexin V and 2.5 µl PI (Beckman Coulter). The cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Then, 400 µl of 1× binding buffer was added to each tube. Analysis was performed by Navios Flow Cytometer (Beckman Coulter) to identify the subpopulations of the apoptotic cells within 1 h. Apoptotic cells were gated and enumerated by identifying those cells that exhibited FITC and PI staining. Annexin V-FITC labeling was used to quantitatively determine the percentage of cells that were undergoing apoptosis. PI was used to distinguish necrotic from non necrotic cells. The biparametric analysis showed three distinct populations: viable cells which had low FITC and low PI signals, apoptotic cells which had high FITC and low PI signals, and necrotic cells which had high FITC and high PI signals. A minimum of 20,000 events were collected on each sample.

## Seeding Cells into xCELLigence Plates

The E16 xCELLigence plates were prepared by addition of complete media (50 µL) to every well. After equilibration to 37 °C, plates were inserted into the xCELLigence station, and the base-line impedance was measured to ensure that all wells and connections were working within acceptable limits. The software automatically informs the researcher if any connection problems arise. Following harvesting and

counting, cells were diluted to the correct seeding density and added to the wells in 50 µL.

### RNA extraction and quantification

Total RNA was extracted using RNasy Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and quantified by spectrophotometry at 260 nm. Total RNA (up to 1 mg) was reverse transcribed using kit iScript cDNA Synthesis (BioRad). The single cycle reaction consisted of 5 minutes at 25°C, 30 minutes at 42°C for to activated reverse transcriptase and 5 minutes at 5°C for to inactivate enzyme.

### Gene expression analysis

After complementary DNA synthesis, quantitative real-time PCR reactions (RT-PCR) were carried out using the CFX96 Real-Time instrument (Bio-Rad Laboratories Inc, Hercules, CA, USA) and kit iQ SYBR Green Supermix (BioRad). The single-tube RT-PCR assays consisted of 1 denaturation cycle at 95°C for 30 s, 45 cycles of amplification at 95°C for 10 s and 60°C for 30 s. Melt curve analysis was performed by ramping products from 65 to 95°C, acquiring fluorescence readings for each degree change. For genes, the fluorescence of the SYBR green dye was determined as a function of the PCR cycle number, giving the threshold cycle (CT) number. The CT values were used to quantify the PCR product, DCT was calculated by subtracting CT (control gene: Actin) from CT (target genes). The DCT value of the control was used arbitrarily as a constant that was subtracted from all other DCT values to determine DDCT value. In treated RTC cells and PBMC samples the relative expression were generated for each sample by calculating $2^{-\Delta\Delta Ct}$ (134).

The following sets of primers were used: NFkB-F: 5’- GCCAACAGATGGCCCATACC -3’; NFkB-R: 5’- TGCTGGTCCCACATAGTTGC -3’; Caspase 1-F: 5'- ACATCCCACAATGGGCTCTG-3'; Caspase 1-R: 5'- TCCACATCACAGGAACAGGC-3'; Caspase 3-F: 5'-TGCATACTCCACAGCACCTG-3'; Caspase 3-R: 5'-TTCTGTTGCCACCTTTCGGT-3'; TNF-α-F: 5'-CCAGACCAAGGTCAACCTCC-3'; TNF-α-R: 5'-CCCTCCCAGATAGATGGGCT-3'; IL-1β-F: 5'-CCACCTCCAGGGACAGGATA-3'; IL-1β-R: 5'-CAACACGCAGGACAGGTACAG-3'; NLRP3-F: 5'-GAGGAAAAGGAAGGCCGACA-3'; NLRP3-R: 5'-CCCGGCAAAAACTGGAAGTG-3'; PYCARD-F: 5'-CCTCAGTCGGCAGCCAAG-3'; PYCARD-R: 5'-GGTACTGCTCATCCGTCA-3'; TLR2-F: 5'-CCCTGGGCAGTCTTGAACAT-3'; TLR2-R: 5'-GGCTTGAACCAGGAAGACGA-3'; TLR4-F: 5'-TCCCCTGAGGCATTTAGGCA-3'; TLR4-R: 5'-GAAAAGGCTCCCAGGGCTAA-3'. The housekeeping gene Actin was amplified in parallel in all amplification sets.

### EVs isolation and count

Plasma EVs from cirrhotic patients and healthy subject were collected from 1 ml of plasma. EVs were isolated from plasma using a two-step differential centrifugation protocol based on a first low-speed centrifugation, 2000 g for 5 minutes, in order to remove cells, cellular debris and apoptotic bodies. The supernatant was subsequently ultracentrifuged at 100,000 g for 1 h at 4°C washed in serum-free medium 199 (M-199) to sediment plasma vesicles. Pellet of EVs was resuspended in M-199 (Sigma Aldrich) and 1% DMSO (Sigma-Aldrich) was added to allow freezing storage in -80°C until use.

To trace EVs by fluorescent microscopy or FACS analysis, EVs were labelled with the red fluorescent aliphatic chromofore intercalating into lipid bilayer PKH26 DYE (Sigma-Aldrich). After labelling, EVs were washed and ultrecentrifuged at 100,000 g for 1 hours at 4°C. EVs pellets were suspended in M-199and stored at -80°C until use.

Plasma EVs were analysed by nanoparticle tracking analysis (NTA), using the NanoSight LM10 System (NanoSight Ltd, Amesbury, UK), configured with a 405 nm laser and a high sensitivity digital camera system as previous described (135). Briefly, EVs coming from 1 ml of plasma were resuspended in 200 µl of M-199 and 1% DMSO. Each samples was then diluted 1:1000 in physiologic solution (Fresenius Kabi, Runcorn, UK). For this analysis, a monochromatic laser beam at 405 nm was applied to the diluted suspension of EVs. For each sample, three videos of 30 seconds duration were recorded and number of particles/ml and mean size was averaged. NTA post-acquisition settings optimized and kept constant between samples, and each video was then analysed to give the mean, mode, and median visicles size together with an estimation of the concentration.

### EVs characterization by FACS

Flow cytometry analysis was performed with a FACSCalibur machine using CellQuest software (Becton Dickinson Bioscience Pharmingen, Franklyn Lake, NJ, USA). As EVs are too small for FACScan analysis, we bound EVs to surfactant-free white aldehyde/sulfate latex beads 4% w/v, 4 mm diameter (Molecular Probes, Invitrogen) (136). We incubated beads with EVs for 30 minutes at RT and then overnight at 4°C in a final volume of 1 ml PBS-BSA 0.5%. Then the adsorbed EVs were divided in different vials and incubated with antibodies diluted 1:50, for 15 minutes at 4°C. The adsorbed EVs were then washed and analyzed with a FACSCalibur and CellQuest software. Flow cytometry was performed using anti-human monoclonal antibodies (MAbs): FITC conjugated CD41(Becton Dickinson, San Jose, CA), CD42b and CD14 (Dako, Copenhagen, Denmark) and PE-conjugated CD62E, CD31, α4 integrin(Becton Dickinson, San Jose, CA), L-selectin (Dako, Copenhagen, Denmark), β1-integrin

(Chemicon Int., Temecula, CA). Comparison of plasma EVs among groups was performed by evaluating the relative amount of EVs positive for a selected marker in respect to all EVs, in conditions where the same quantity of EVs was tested (concentration detected by NTA analysis).

### Viability and Proliferation Assays on RTCs

RTCs were cultured on 24-well plates (Falcon Labware, Oxnard, CA) at a concentration of $5 \times 10^4$ cells/well and incubated with $5 \cdot 10^8$ EVs/ml for 24h and 250 μg/ml XTT (Sigma,St. Louis, MO) in a medium lacking phenol red. The absorption values at 450 nm (XTT) were measured in an automated spectrophotometer at different time points. All experiments were performed in triplicate.

### Detection of Apoptosis - TUNEL Assay *in vitro* on RTCs

RTCs were subjected to TUNEL assay (terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labelling) (ApopTag, Oncor, Gaithersburg, MD) after starving for 12 hrs without FCS and subsequent incubation with stimulation with EVs for 24h. Then, cells were fixed in 1% paraformaldehyde, post-fixed in pre-cooled ethanol and acetic acid 2:1, incubated with TdT enzyme in a humidified chamber at 37° C for 1 hr and counterstained with antidigoxigenin-FITC antibody and with propidium iodide (1 μg/mL). Samples were analyzed under a fluorescence microscope and green-stained. Apoptotic cells were counted in 10 non-consecutive microscopic fields.

### ROS detection

Image-iT LIVE Green Reactive Oxygen Species (ROS) Detection Kit was used to analyze oxidative stress on RTCs as suggested by manufacturer (Life Technologies). Briefly, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) was

added to RTCs in different experimental conditions: after 30 min, cells were fixed with 4% paraformaldehyde and then counterstained with Hoechst and analyzed by FACS analysis.

### Detection of FITC-conjugated albumin uptake

Albumin uptake was studied after incubation of RTCs previously exposed to EVs with 50 µg/ml of FITC-conjugated human albumin (Sigma, St. Louis, MO, USA) at 37°C for two hours. After FITC-albumin challenge, RTCs were extensively washed twice with PBS, detached from tissue culture plates with EDTA and analysed by FACS.

### FACS analysis

For FACS analysis, after exposure to EVs, RTCs were detached from tissue culture plates with EDTA, washed twice with one times PBS and fixed with 4% paraformaldehyde. Cells were then stained for one hour at 4°C with antibodies directed to human zonula occludens-1 (ZO-1), megalin, PGC-1alpha (Santa Cruz Biotech, Santa Cruz, CA, USA). After incubation with primary antibodies, samples were washed twice with PBS and incubated with appropriated FITC-conjugated secondary antibodies (Sigma Aldrich, MO, USA) for 30 minutes at room temperature. At the end of staining, cells were newly washed and subjected to FACS analysis (Becton Dickinson, Franklin Lakes, NJ, USA). All incubation periods were performed using PBS containing 0.25% BSA, 0.1% saponine and 0.0016% sodium azide.

### Statistical analysis

Statistical analysis was performed with GraphPad Prism (Prism 5 for Windows, Version 5.01). Continuous variables are presented as median (25%–75% percentile), according

to their distribution. The difference between 3 or more groups of these variables was analyzed with One-way ANOVA and Kruskal-Wallis test. The difference between two groups was analyzed with t-test. Significance level for all tests was set at $p<0.05$.

# Results

## Patients

In the first part of the study, 22 patients were enrolled: 12 patients with compensated cirrhosis, 8 with acute decompensation of cirrhosis and 2 with acute-on-chronic liver failure. In **Table 2** demographic and biochemical data about the three different groups have been reported.

**Table 2:** Demographic and biochemical data of patients included. Data are expressed as median and range. Differences between groups were analyzed using ANOVA test.

| | range | Compensated Cirrhosis | Acute Decompensation | Acute-on-chronic | *p-value* |
|---|---|---|---|---|---|
| male (%) | | 77% | 75% | 50% | |
| age (y) | | 63 (53-73) | 61 (51-64) | 59 (55-64) | ns |
| Total bilirubin (µmol/L) | 1.7-17.0 | 14.3 (9.3-20.0) | 80.3 (32.4-124.0) | 590.8 (501.6-679.9) | <0.0001 |
| Direct bilirubin (µmol/L) | 0.0-3.4 | 6.8 (4.4-8.8) | 58.9 (21.8-86.6) | 477.0 (407.2-546.7) | <0.0001 |
| INR | 0.88-1.13 | 1.1 (1.0-1.1) | 1.4 (1.3-1.7) | 1.9 (1.8-1.9) | 0.001 |
| Creatinine (µmol/L) | 62-115 | 72.0 (65.0-92.0) | 67.0 (60.0-84.5) | 204.0 (150.0-258.0) | ns |
| Urea (mmol/L) | 2.50-7.50 | 5.6 (4.3-7.2) | 6.7 (4.8-9.6) | 27.5 (19.7-35.2) | ns |
| Na (mmol/L) | 136-145 | 140 (139-140) | 133 (130-135) | 132 (129-126) | 0.001 |
| K (mmol/L) | 3.4-4.5 | 4.1 (4.0-4.2) | 3.8 (3.7-4.4) | 4 (3.9-4.2) | ns |
| White blood cells (*10.9/L) | 4.40-11.00 | 5.3 (4.5-7.7) | 6.6 (4.7-9.1) | 15.6 (11.6-19.6) | ns |
| Red blood cells (*10.12/L) | 4.50-5.90 | 4.9 (4.3-5.0) | 3.0 (2.8-3.3) | 3.4 (3.2-3.7) | <0.0001 |
| Haemoglobin (g/dL) | 14-17.5 | 13.8 (12.5-14.4) | 9.7 (9.3-10.3) | 12.0 (10.3-13.6) | 0.002 |
| Platelets (*10.9/L) | 150-450 | 148.5 (70.8-201.2) | 78.0 (55.5-126.0) | 59 (59-59) | ns |
| AST (U/L) | 10-45 | 31 (25-50) | 51 (41-64) | 43 (43-43) | ns |
| ALT (U/L) | 10-50 | 30 (22-44) | 32 (24-37) | 31 (31-31) | ns |
| GGT (U/L) | 3-65 | 32 (21-45) | 37 (30-46) | 99 (99-99) | ns |
| ALP (U/L) | 56-128 | 75 (63-99) | 124 (103-166) | 250 (250-250) | 0.006 |
| Albumin (g/L) | 1-47 | 43 (41-44) | 29 (25-31) | 30 (30-30) | 0.003 |

### Inflammatory profile in patients

Plasma inflammatory cytokines levels were measured by ELISA assay in all patients. IL-6, IL-1β and IL-18 levels did not show significantly differences between the groups (**Table 3**). Unfortunately, the number of patients whit acute decompensation of cirrhosis and acute-on-chronic groups was really low.

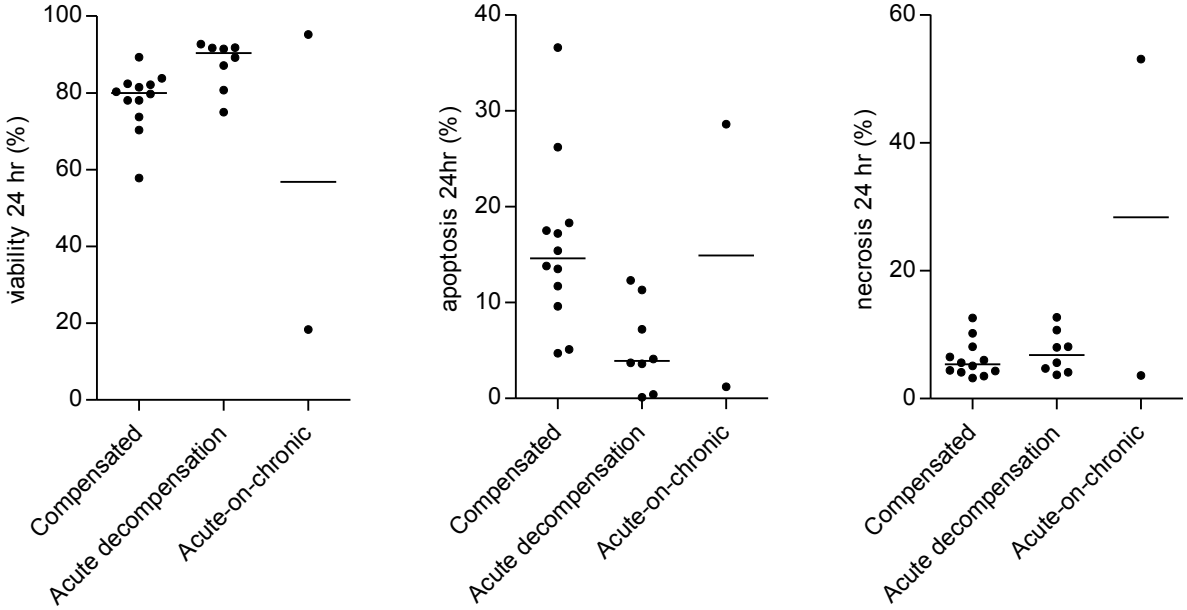
**Table 3**: IL-6, IL-1β and IL-18 levels in plasma of patients detected with ELISA assay. Data are expressed as median and range. Differences between groups were analyzed using ANOVA test.

| | Compensated cirrhosis | Acute decompensation | Acute-on-chronic | *p-value* |
|---|---|---|---|---|
| **IL-6 (pg/ml)** | 32.5 (27.4-47.1) | 41.1 (31.3-58.5) | 42.24 | 0.631 |
| **IL-1β (pg/ml)** | 9.6 (9.4-10.1) | 10.3 (8.8-11.1) | 10.11 | 0.827 |
| **IL-18 (pg/ml)** | 187 (138.1-220.2) | 292.4 (167.9-408.6) | 263.6 | 0.318 |
| Statistical analysis: non-parametric Kruskal Wallis test for n-indipendent samples | | | | |

## Effect of plasma on RTCs viability

Cytotoxic effect of plasma from patients was assayed in RTCs by flow-cytometry according to (137). The RTCs incubated with 10% v/v of plasma from patients with compensated cirrhosis, acute decompensation of cirrhosis and acute-on-chronic liver failure did not show significantly different levels of viability, apoptosis and necrosis after 24 hrs of stimulation (**Figure 4**).

**Figure 4**: cell viability, apoptosis and necrosis detected by FACS after 24 hours of stimulation with plasma from the three groups of patients enrolled. Data are represented as single values and median.



**Gene expression analysis on treated RTCs**

After stimulation, RTCs were collected for gene expression analysis. Total RNA was extracted and expression of the main molecules of inflammasomes (NFkB, NLRP3, Caspase-1 and -3, TNF-α, PYCARD and IL-1β) was assayed. The expression levels of molecules of inflammasome was not significantly different between the groups (**Table 4**).

**Table 4**: gene expression in RTCs stimulated with plasma from patients of the three groups enrolled. Data are expressed as median and range. Differences between groups were analyzed using ANOVA test.

| | **Compensated cirrhosis** | **Acute decompensation** | **Acute-on-chronic** | ***p-value*** |
|---|---|---|---|---|
| **NFkB** | 1120 (1040-1528) | 960 (728-1079) | 924 (833-1016) | 0.354 |
| **NLRP3** | 1.7 (1.5-1.8) | 1.0 (0.9-1.2) | 1.2 (1.2-1.2) | 0.159 |
| **Caspase-1** | 27.7 (20.0-28.5) | 9.1 (4.9-13.2) | 2.1 (1.6-2.6) | 0.056 |
| **TNF- α** | 0.3 (0.3-0.3) | 0.1 (0.1-0.3) | 0.4 (0.3-0.5) | 0.493 |
| **PYCARD** | 2.7 (2.3-2.8) | 2.7 (2.1-3.6) | 1.4 (1.4-1.5) | 0.096 |
| **IL-1β** | 3.3 (2.6-3.8) | 1.5 (1.1-3.6) | 0.3 (0.2-0.3) | 0.073 |
| **Caspase-3** | 2.3 (2.2-2.3) | 1.0 (0.9-1.9) | 0.7 (0.6-0.8) | 0.254 |
| The data are reported as 2-ΔΔCT<br>Statistical analysis: non-parametric Kruskal Wallis test for n-indipendent samples | | | | |

**Gene expression analysis on PBMC from patients**

Gene expression analysis of molecules of inflammasomes and the two main receptors of PAMPS and DAMPS (TLR2 and 4) was assayed also in PBMC extracted from whole blood of patients. No differences in gene expression of molecules of inflammasome were found, except for TLR-2 (**Table 5**). Its expression in PBMC from patients with acute decompensation was higher than compensated cirrhosis (p=0.031).

**Table 5**: gene expression in PBMC from patients of the three groups enrolled. Data are expressed as median and range. Differences between groups were analyzed using ANOVA test.

| | Compensated cirrhosis | Acute decompensation | Acute-on-chronic | *p-value* |
|---|---|---|---|---|
| **NFkB** | 0.0014 (0.0011-0.0025) | 0.0007 (0.0003-0.0012) | 0.0011 (0.0010-0.0012) | 0.391 |
| **NLRP3** | 0.0008 (0.0005-0.0011) | 0.0004 (0.0003-0.0005) | 0.0006 (0.0005-0.0007) | 0.441 |
| **Caspase-1** | 0.16 (0.15-0.35) | 0.10 (0.06-0.15) | 0.08 (0.06-0.09) | 0.208 |
| **TNF- α** | 0.0019 (0.0016-0.0091) | 0.0006 (0.0003-0.0011) | 0.0005 (0.0005-0.0006) | 0.077 |
| **PYCARD** | 0.075 (0.062-0.120) | 0.065 (0.036-0.078) | 0.043 (0.034-0.051) | 0.441 |
| **IL-1β** | 0.0026 (0.0021-0.0034) | 0.0015 (0.0007-0.0022) | 0.0013 (0.0010-0.0016) | 0.441 |
| **Caspase-3** | 0.0026 (0.0021-0.0037) | 0.0012 (0.0011-0.0027) | 0.0022 (0.0020-0.0023) | 0.510 |
| **TLR-2** | 0.0043 (0.00021-0.015) | 0.016 (0.011-0.030) | 0.0071 (0.0070-0.014) | 0.036* |
| **TLR-4** | 0.000025 (0.0000061-0.0018) | 0.0013 (0.000081-0.0021) | 0.00080 (0.00061-0.0012) | 0.422 |
| The data are reported as 2-ΔΔCT<br>Statistical analysis: non-parametric Kruskal Wallis test for n-indipendent samples | | | | |

## Evaluation of cell viability by xCELLigence Real-Time Cell Analyzer

In a second phase of the study we included healthy subjects as control groups and we incremented the number of patients with acute-on-chronic liver failure (n=5).

Cytotoxicity assay was performed with the xCELLigence Real-Time Cell Analyzer (RTCA) in order to measure RTCs viability and proliferation in real time after stimulation with plasma of patients and controls. It was assayed only a small group of patients for setting-up. In **Figure 5** data about percentage of cell viability after 24 hrs of stimulation with 10% v/v of plasma has been reported. After these first experiments it was clear that there was not a defined trend in viability rate in the different groups.

The same experiment was performed adding albumin to cell medium with 10% v/v of plasma from patient. It was used solution containing 0-20-45 g/L of albumin. **Figure 6**

shows that physiologic concentration of albumin (45 g/L) reduced cell death respect the absence of albumin in medium.

**Figure 5**: viability rate in cells stimulated for 24 hours with plasma from patients and healthy subject enrolled. Data are represented as single values and median.

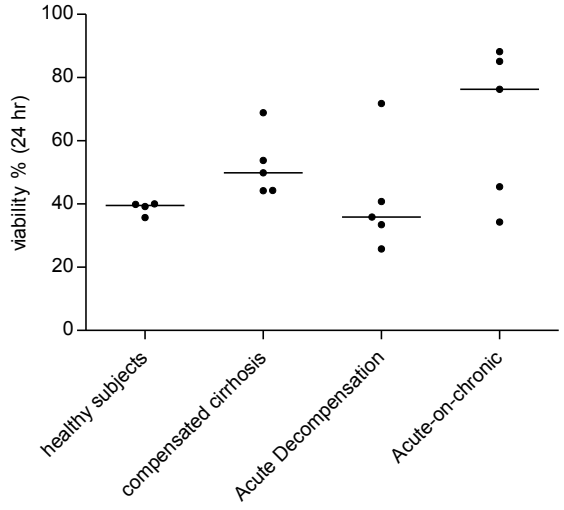
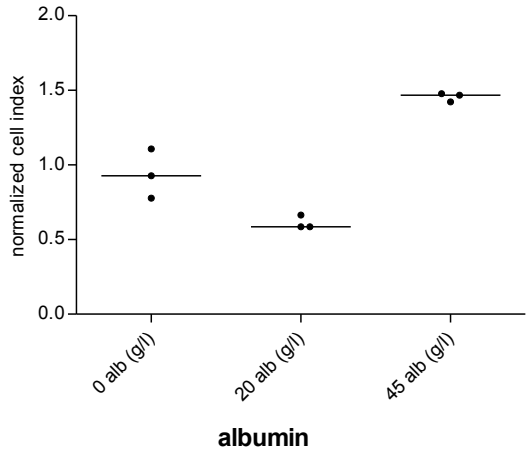



**Figure 6**: viability, represented as normalized cell index, in cell treated with plasma from one patients in medium containing different concentration of albumin. Data are expressed as single values and median.



## Characterization of extracellular vesicles in cirrhotic patients

### Patients

EVs analysis was carried on 48 patients: 12 healthy subjects, 12 patients with compensated cirrhosis, 13 patients with acute decompensation in cirrhosis and 11 patient with acute-on-chronic liver failure. **Table 5** record demographic and biochemical data of all patients. As expected there was a stepwise increase in bilirubin, INR, serum creatinine, white blood cells and haemoglobin moving from compensated cirrhosis to acute decompensation and acute-on-chronic liver failure.

**Table 5:** Demographic and biochemical data of patients included. Data are expressed as median and range. Differences between groups were analyzed using ANOVA test.

| | **Compensated Cirrhosis** | **Acute Decompensation** | **Acute-on-chronic** | ***p-value*** |
|---|---|---|---|---|
| male (%) | 75% | 53.8% | 81.8% | |
| age (y) | 61.5 (52.5-70.5) | 51.0 (43.8-65.3) | 54.0 (51.0-66.0) | ns |
| Total bilirubin (µmol/L) | 26.0 (14.2-35.3) | 54.6 (33.5-138.2) | 300.2 (151.1-434.4) | <0.0001 |
| Direct bilirubin (µmol/L) | 10.5 (6.3-13.0) | 38.8 (22.1-103.3) | 244.5 (117.0-356.3) | <0.0001 |
| INR | 1.3 (1.0-1.3) | 1.5 (1.4-1.6) | 1.8 (1.7-2.8) | <0.0001 |
| Creatinine (µmol/L) | 69.5 (59.8-86.0) | 68.0 (52.5-79.0) | 117.0 (73.0-312.0) | 0.017 |
| Urea (mmol/L) | 4.3 (3.7-7.5) | 3.8 (2.9-8.7) | 11.6 (8.7-26.5) | 0.0024 |
| Na (mmol/L) | 140 (137-141) | 135 (127-137) | 133 (130-137) | 0.0198 |
| K (mmol/L) | 4.3 (4.0-4.4) | 3.7 (3.3-4.2) | 4.3 (3.7-4.7) | ns |
| White blood cells (*10.9/L) | 3.7 (2.8-4.3) | 5.8 (4.1-6.6) | 10.0 (6.8-11.8) | 0.0008 |
| Red blood cells (*10.12/L) | 4.6 (4.3-4.9) | 2.8 (2.7-3.6) | 3.0 (2.5-3.5) | <0.0001 |
| Haemoglobin (g/L) | 125 (102-149) | 101 (87-105) | 95.0 (83-116) | 0.0298 |
| Platelets (*10.9/L) | 53.0 (38.0-120.3) | 95.0 (78.0-134.0) | 59.0 (43.0-65.0) | 0.0242 |
| AST (U/L) | 41.0 (29.3-89.0) | 67.0 (41.8-82.8) | 118.0 (50.0-215.0) | ns |
| ALT (U/L) | 30.0 (18.8-100.3) | 34.0 (17.0-44.0) | 58.0 (31.0-128.0) | ns |
| GGT (U/L) | 37.5 (18.8-103.3) | 66.0 (44.5-378.0) | 48.5 (14.0-95.3) | ns |
| ALP (U/L) | 86.5 (54.3-104.8) | 156.0 (118.0-165.0) | 138.0 (98.5-221.0) | 0.0057 |
| Albumin (g/L) | 40.0 (38.0-46.0) | 28.0 (24.0-36.0) | 29.0 (26.0-32.0) | 0.0005 |

### Detection of plasma EVs in cirrhotic patients and healthy subjects

EVs in plasma obtained from all enrolled patients and healthy subjects were quantified and size was determined by NanoSight analysis. Data collected about size and concentration were reported in **Table 7**.

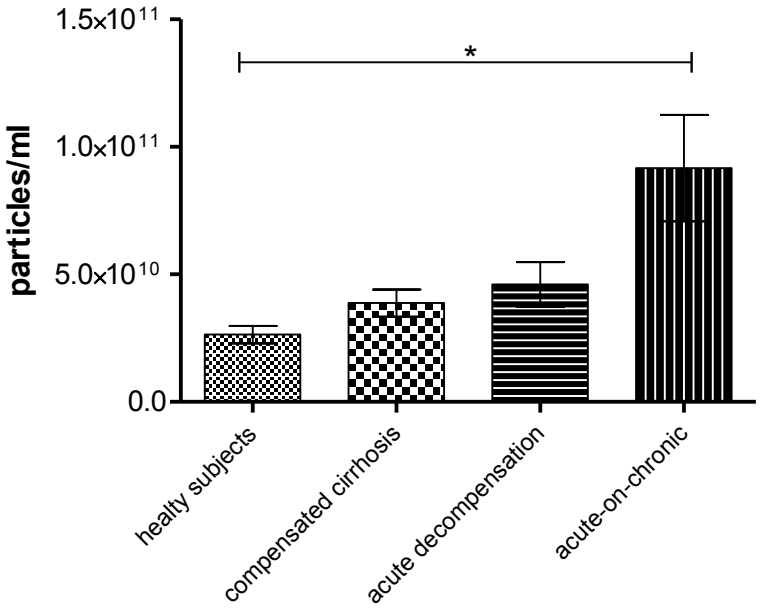
Plasma EVs showed a heterogenic size between the groups: the size of EVs from healthy subjects was significantly lower only respect Acute-on-chronic group (p=0.0019).

**Table 7**: size and concentration of EVs analyzed in the four groups. Data are reported as median of mode values calculated by Nanosight, and range.

| samples | Mode size (nm) | Concentration (particles/ml) (*$10^{11}$) |
|---|---|---|
| healthy subject | 167,5 (130,6-192,1) | 0,2 (0,2-0,3) |
| compensated cirrhosis | 191,0 (163,2-238,9) | 0,4 (0,3-0,5) |
| Acute Decompensation | 189,9 (174,4-223,0) | 0,3 (0,2-0,7) |
| Acute-on-chronic | 220,6 (193,3-250,9) | 0,7 (0,5-1,5) |

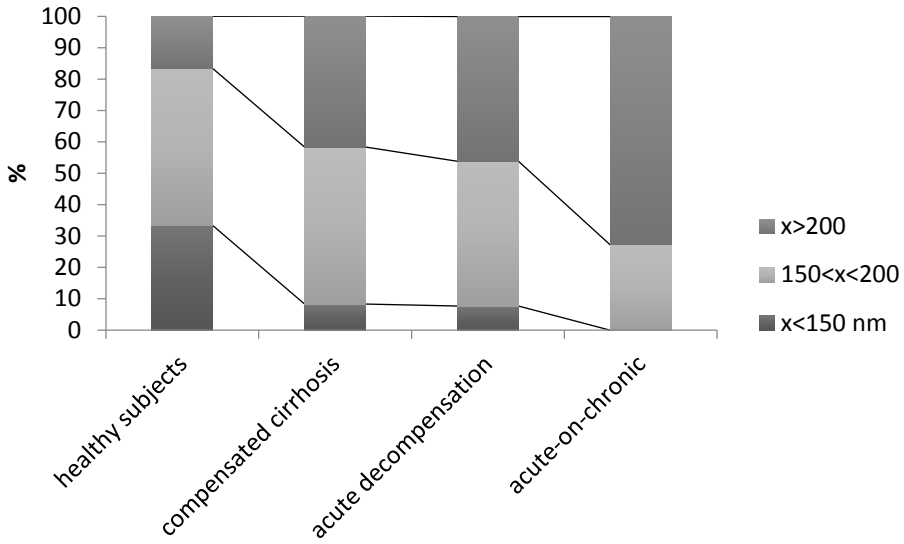
Data about concentration of EVs showed a trend toward their increase in patients with cirrhosis and ACLF, as shown in **Figure 7**. In fact the concentration of EVs from Acute-on-chronic group was significantly higher than concentration in healthy subjects (p=0.0021).

**Figure 7**: histogram of concentrations (in particles for milliliter) of EVs extracted from plasma of patients enrolled. Data are represented as mean ± standard error of the mean (SEM). Statistical analysis was made with ANOVA test.




We then stratified any group of patients according to the most frequent size of EVs as follows: "less than 150 nm", "between 150 and 200 nm" and "up to 200 nm". As shown in **Figure 8**, the percentage of patients having a prevalence of small vesicles was higher in healthy subjects and disappeared in patients with acute-on-chronic liver failure. Controversely, the percentage of big vesicles was higher in acute-on-chronic liver failure group than the other groups.

**Figure 8**: in each group of subject enrolled, EVs is grouped by size. Healthy subject have EVs smaller than 200 nm. Acute-on-chronic patients have EVs bigger than 150 nm.




## Characterization of EVs

Cytofluorimetric analysis of the EVs using the antibody showed in **Table 8** was used for determining the origin of EVs.

**Table 8**: selected markers for EVs origin characterization

| Marker | Localization | Function |
|---|---|---|
| **CD41** | platelets | membrane glycoprotein |
| **CD42b** | platelets | Von Willebrant factor Receptor |
| **CD14** | Monocyte, macrophages, granulocytes | membrane receptor |
| **CD31/PECAM-1** | endothelium | cell adhesion |
| **CD62E/E-selectin** | platelets activated endothelium | cell adhesion |

Analysis of EVs showed the absence of typical platelet markers such as CD41 and CD42b and also for monocyte/macrophages/granulocyte marker CD14 (**Figure 9 A, B, C**). The endothelial marker CD31 was expressed in EVs from patients with Acute Decompensation of cirrhosis and Acute-on-chronic liver failure (**Figure 9 D**). EVs from Acute-on-chronic patients were positive for CD42E, a platelet activated endothelium

marker, and this expression was significantly higher respect healthy subjects and patients with compensated cirrhosis (p=0.0041 and p=0.0111, respectively)(**Figure 9 E**).

EVs were characterized also for the presence of molecules involved in vesicles internalization in target cells or tissue (integrins and selectins) and a molecule crucial for cell signaling in both adaptive and innate immunity, CD40 Ligand (**Table 9**).

**Table 9:** Other selected markers for EVs characterization

| Marker | Localization | Function |
|---|---|---|
| **CD40 Ligand/CD154** | T activated cells | cytokines production, macrophages activation |
| **CD29/β1-integrin** | focal adhesion | cell adhesion |
| **α-4 integrin** | leukocytes | cell adhesion |
| **CD62/L-selectin** | white blood cells | hinder MV endocitosis |

α4 , β1-integrin and L-selectin expression was higher in EVs from acute-on-chronic liver failure patients than healthy subjects (all p=0.0017). L-selectin was more expressed in patients with acute-on-chronic liver failure than in those with compensated cirrhosis (p=0.0014)(**Figure 9 F, G, H**). Expression of CD40 Ligand was higher in all group of patients than healthy subject (healthy vs compensated p=0.0009; healthy vs acute decompensation p=0.0226; healthy vs acute-on-chronic p<0.0001) and between patients, the expression was higher in acute-on-chronic liver failure group than in compensated cirrhosis (p=0.0008)(**Figure 10**).

**Figure 9**: Graphs represent EVs characterization. Comparison of EVs among groups was performed by evaluating the relative amount of EVs positive for a selected marker in respect to all EVs. Data are shown as single values, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.

**Figure 9**

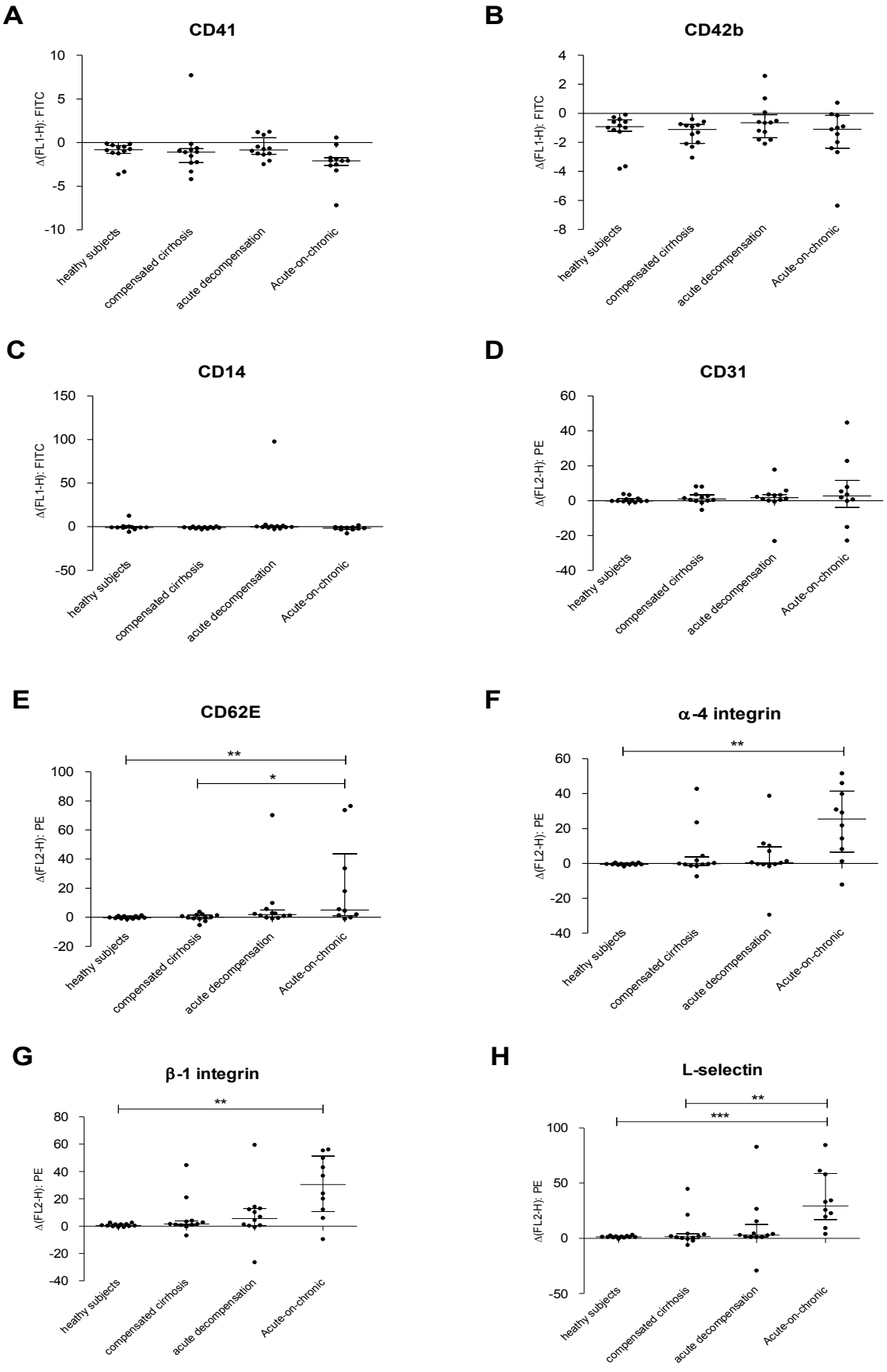
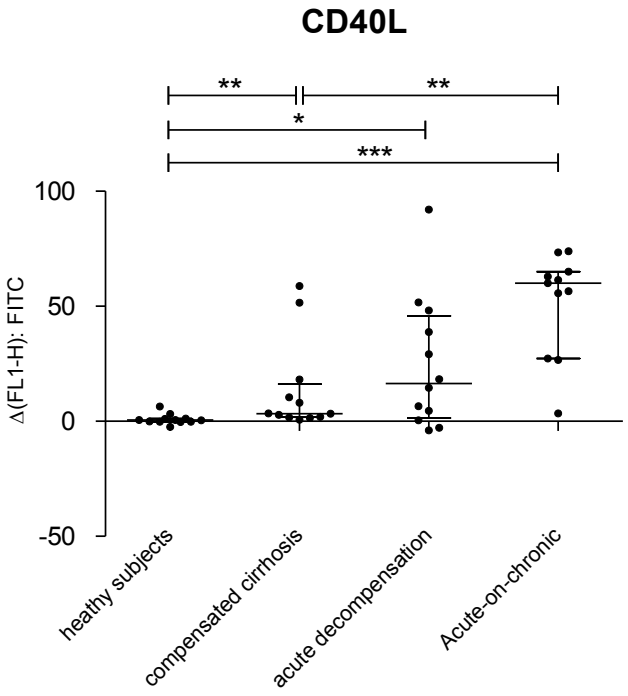
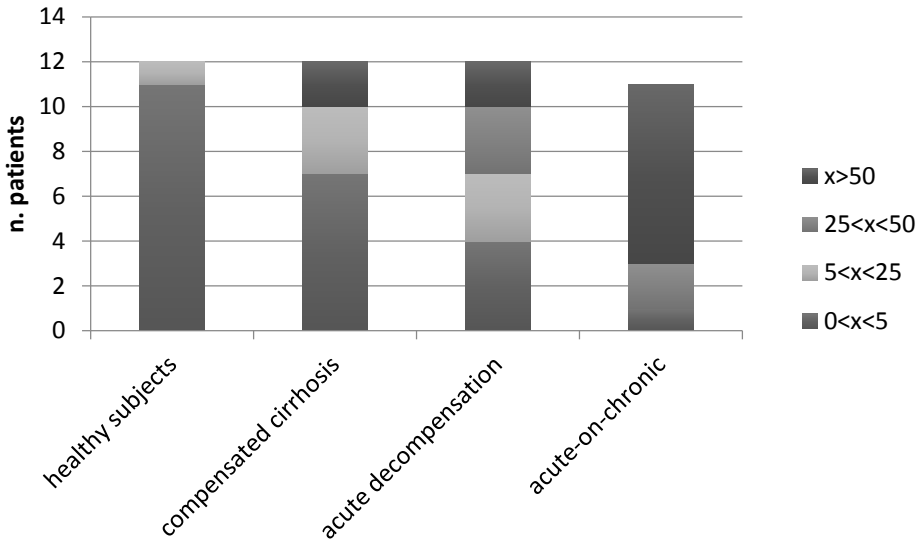

A
CD41
Δ(FL1-H): FITC
10
5
0
-5
-10
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
B
CD42b
Δ(FL1-H): FITC
4
2
0
-2
-4
-6
-8
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
C
CD14
Δ(FL1-H): FITC
150
100
50
0
-50
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
D
CD31
Δ(FL2-H): PE
60
40
20
0
-20
-40
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
E
CD62E
**
*
Δ(FL2-H): PE
100
80
60
40
20
0
-20
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
F
α-4 integrin
**
Δ(FL2-H): PE
60
40
20
0
-20
-40
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
G
β-1 integrin
**
Δ(FL2-H): PE
80
60
40
20
0
-20
-40
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic
H
L-selectin
**
***
Δ(FL2-H): PE
100
50
0
-50
heathy subjects
compensated cirrhosis
acute decompensation
Acute-on-chronic

**Figure 10:** CD40L expression in EVs for each group. Comparison of EVs among groups was performed by evaluating the relative amount of EVs positive for a selected marker in respect to all EVs. Statistical analysis was performed using the Mann-Whitney test.




When we divided patients in groups according to CD40 Ligand expression percentage, patients with higher level of CD40 Ligand expression (>50%) were almost completely acute-on-chronic, instead healthy subjects expressed lower levels of this molecule (<5%)(**Figure 11**).

**Figure 11:** expression levels of CD40L in EVs is categorized in the histogram. Classes are: CD40L levels lower than 5% in EVs, levels between 5 and 25%, between 25 and 50% and expression levels above 50% in EVs. In ordinate axis is represented the number of patients.

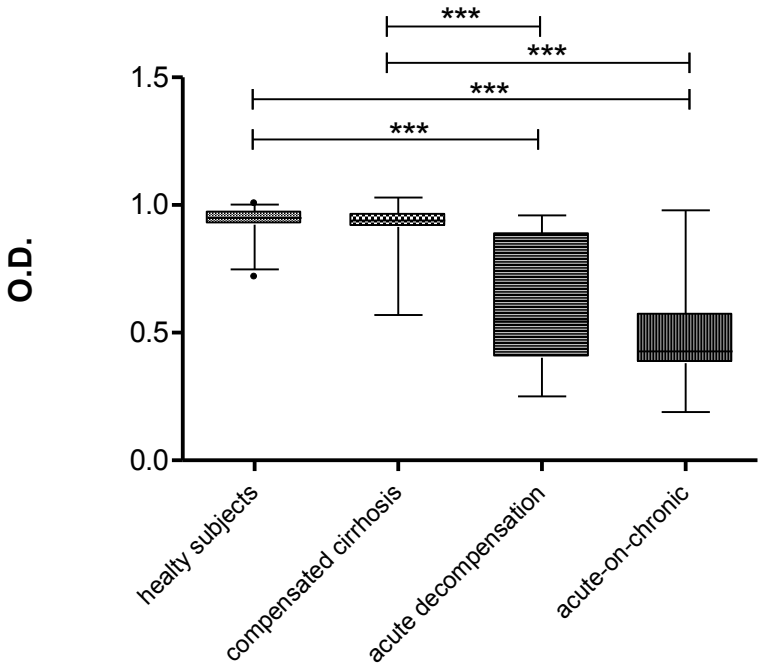

14
12
10
8
6
4
2
0
n. patients
healthy subjects
compensated cirrhosis
acute decompensation
acute-on-chronic
x>50
25<x<50
5<x<25
0<x<5

**EVs: *In vitro* experimentation**

**Effect of EVs on RTC viability**

EVs cytotoxic effects *in vitro* was tested on RTCs (**Figure 12**). After 48 hr of incubation, cells showed decrease in cell viability at MTT assay when incubated with EVs from patients with acute decompensation and acute-on-chronic respect controls and EVs from patients with compensated cirrhosis (all p<0.0001).

**Figure 12**: Effect of EVs on RTCs viability, expressed as optical density (O.D.). Less is O.D. lower is cell viability. Statistical analysis was performed using the Mann-Whitney test.




Apoptosis was detected on RTCs stimulated with EVs by TUNEL assay (**Figure 13**). Apoptotic rate increased in cells incubated with EVs from patients than in those incubated with EVs from healthy subject (all p<0.0001). Apoptotic rate was significantly higher in acute-on-chronic liver failure group than in compensated cirrhosis group (p=0.0006).

Cell stimulation with EVs led to massive production of ROS when vesicles belonging to patients with acute decompensation or acute-on-chronic liver failure were used ($p<0.0001$). Instead, there was no significant ROS production from cells incubated with EVs from patients with compensated cirrhosis respect healthy subjects (**Figure 14**).

**Figure 13**: apoptosis in RTCs stimulated with EVs assayed with TUNEL. In order axis there is the mean number of apoptotic cells detected for field. Data is represented as single value, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.

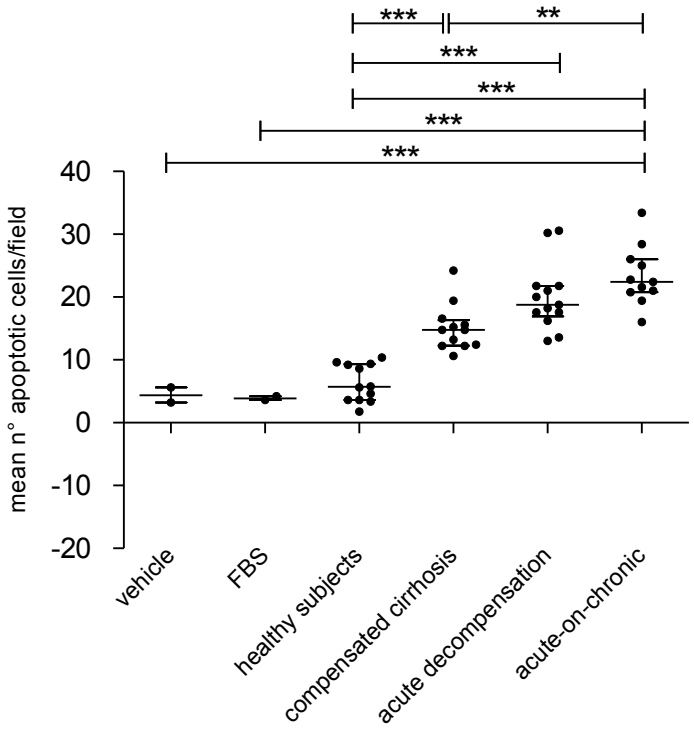
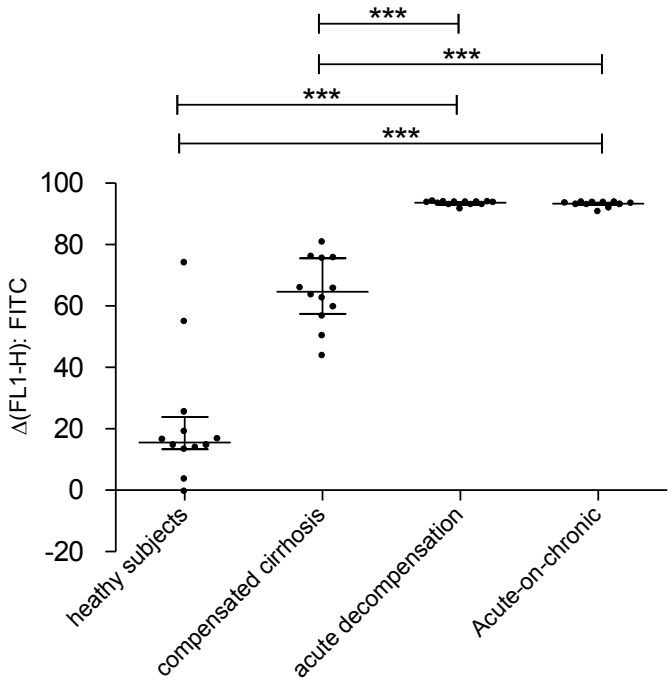


**Figure 14**: ROS production in RTCs incubated with EVs. Comparison of ROS among groups was performed by evaluating the relative amount of cells positive for a selected marker in respect to all cells. Data is represented as single value, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.




**Effect of EVs on cellular functions and activity**

Using Albumin-fitc it was possible to track transportation of albumin inside epithelial tubular cells. **Figure 15** shown a decrease in albumin transportation in cells incubated with EVs from patients with acute decompensation and acute-on-chronic liver failure ($p<0.0001$). One of albumin transporters, Megalin, was detected by flow cytometry assay and its expression didn't change between the groups (**Figure 16**).

**Figure 15**: albumin-fitc intake in RTCs incubated with EVs. Comparison of albumin amount in cells among groups was performed by evaluating the relative amount of cells positive for albumin in respect to all cells. Data is represented as single value, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.

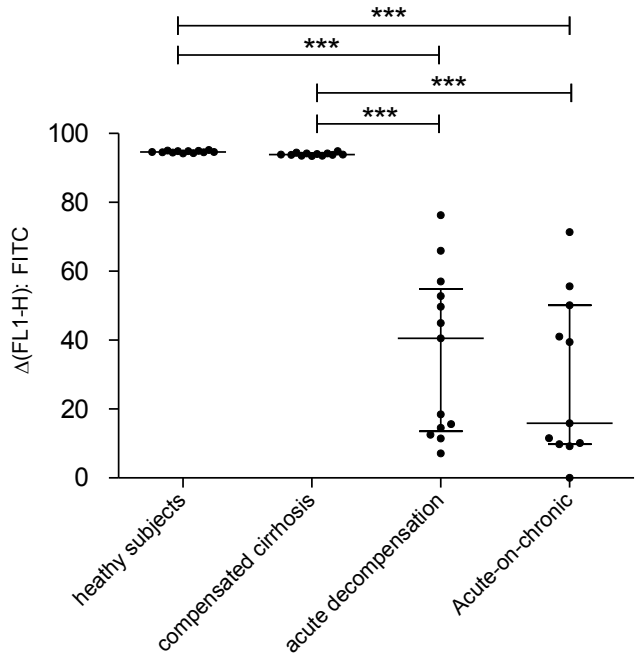
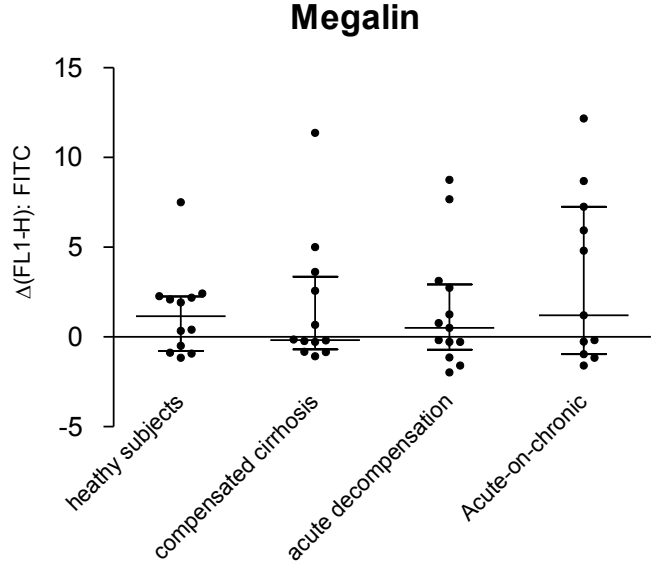
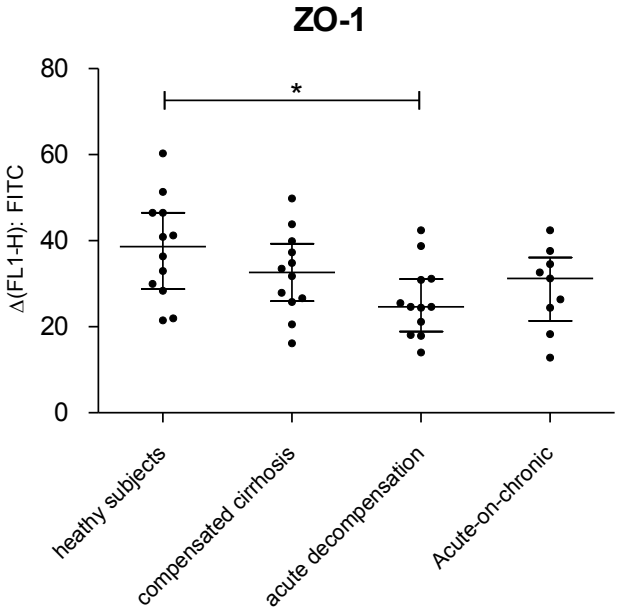



**Figure 16**: Megalin expression in RTCs incubated with EVs. Comparison of Megalin expression in cells among groups was performed by evaluating the relative amount of cells positive for Megalin in respect to all cells. Data is represented as single value, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.



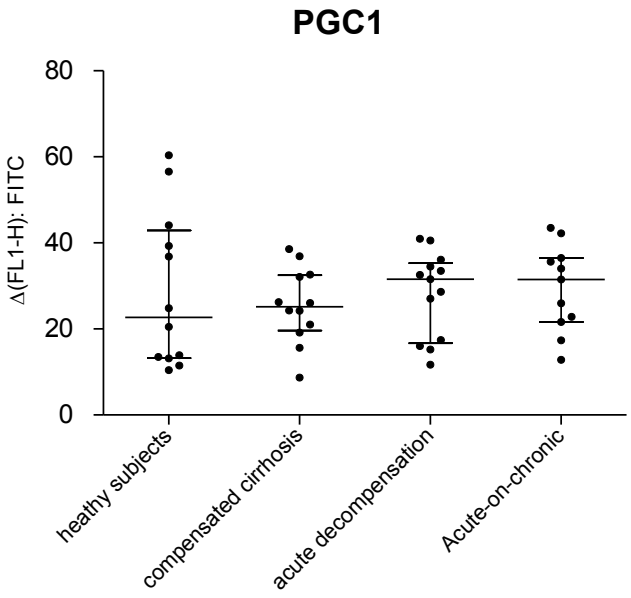
The expression of cell-cell junction protein ZO-1 decreased in cells incubated with EVs from patients than controls, but a significant difference was found only between patients with acute decompensation and healthy subjects (p=0.0166) (**Figure 17**).

**Figure 17**: ZO-1 expression in RTCs incubated with EVs. Comparison of ZO-1 expression in cells among groups was performed by evaluating the relative amount of cells positive for ZO-1 in respect to all cells. Data is represented as single value, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.




It was determined also the expression of PGC1α, a molecule involved in mitochondria biogenesis, in renal tubular cells stimulated with EVs. As shown in **Figure 18** there was no difference in PGC1α expression between the groups.

**Figure 18**: PGC1α expression in RTCs incubated with EVs. Comparison of PGC1α expression in cells among groups was performed by evaluating the relative amount of cells positive for PGC1α in respect to all cells. Data is represented as single value, median and interquartile range. Statistical analysis was performed using the Mann-Whitney test.



## Discussion

In this project it was studied the acute and acute-on-chronic liver failure from a biological point of view. Starting from the patients, it was carry on a study with different approaches, in order to *in vitro* characterize liver disease. The first approach was focused on the characterization of inflammatory profile of the patients through the detection of pro-inflammatory cytokines in plasma; the second hypothesized a cytotoxic activity of molecules released in the blood flow from the organ or organs injured. The last one was born on the need of focusing the attention on a single component of plasma, the extracellular vesicles.

Patients enrolled in the first part of the study showed abnormalities in laboratory findings including elevated serum bilirubin, prolonged prothormbin time (elevated international normalized ratio, INR) and hyponatremia. The levels in these parameters significantly differed between the groups of cirrhotic patients, acute decompensation and patients with acute-on-chronic liver failure. Groups differed also for ALP levels and serum albumin concentration. Creatinine and urea, markers of kidney function, were elevated respect physiological range in patients with acute-on-chronic liver failure. Renal dysfunction is critical in the definition of acute-on-chronic liver failure and it has been found a powerful predictor of mortality in patients with cirrhosis. Indeed, these patients have a high risk to develop AKI. AKI is defined as an abrupt (within 48 hours) reduction in kidney function currently defined as an absolute increase in serum creatinine of more than or equal to 0.3 mg/dl (≥ 26.4 μmol/l), or a percentage increase in serum creatinine of more than or equal to 50% from baseline which is known, or presumed, to have occurred whithin the prior 7 days (138, 139). AKI can be found in 27–49% of patients hospitalized for an acute decompensation of cirrhosis (140-143).

AKI markedly increased mortality in cirrhotic patients, whose 3-month mortality rate reached 26% (144). Increasing evidences show that inflammatory response is involved in apoptosis and cellular dysfunction during AKI, mediated by the activation of inflammasome (145). Furthermore, also acute decompensation and acute-on-chronic liver failure were characterized by systemic inflammation (4) and activation of inflammasome (23). For these reasons, first step of the study was to characterize the inflammatory profile of patients through the detection of plasma levels of IL-6, IL-1β and IL-18. The second one was to assess the cytokines release from cells in which inflammasome was activated. Unfortunatly, this determination was difficult to perform because of the small sample size, especially for acute-on-chronic liver failure group. However, these samples were part of a group of 522 patients with decompensated cirrhosis (237 with acute-on-chronic liver failure) and 40 healthy subjects in which it was measured a panel of 29 cytokines and marker of systemic oxidative stress by Clària J. and co-workers and recently published in Hepatology (20). It was shown that patients with acute decompensation and without acute-on-chronic liver failure had very high baseline levels of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-8, macrophage chemotactic protein 1 (MCP-1), inducible protein 10 (IP10), macrophage inflammatory protein(MIP)-1β, granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). Moreover, patients with acute-on-chronic liver failure showed higher levels of pro-inflammatory cytokines than those without. Surprisingly, Clària J. described also a characteristic cytokines profiles according to the type of precipitating event of acute-on-chronic liver failure. Finally, in the paper was highlighter the pivotal role of systemic inflammation as the primary driver of acute-on-chronic liver failure (20). Few years ago, Baroja-Mazo and

colleagues (146) found that activation of inflammasomes led to the release of functional oligomeric inflammasome particles containing both NLRP3 and ASC that acted as danger signals to amplify inflammation by promoting the activation of caspase-1 extracellularly and in surrounding macrophages following internalization of the particles. In macrophages, caspase-1 induces the specific type of cell death called 'pyroptosis'. It was clear that this effect hit not only macrophages but overexpression of ASC was found able to induce the death also in culture of monkey kidney cells (146). For this reason, one aim of this study was to determined the cytotoxic effect of plasma from patients enrolled on renal epithelial cell line. First of all, the effect of circulating molecules in activation of inflammasome in cells was evaluated by gene expression of inflammasome components (receptors, catalytic domain, adaptor molecule, etc..) in epithelial cell line. Secondly, the cell death as a consequence of inflammasome activation after stimulation of cell culture with plasma from patients was assessed. This was performed by detection of annexin V and propidium iodide bond in cells. This approach has been largely applied for understanding the mechanism of cell death involved in Cardiorenal Syndromes pathophysiology (137, 147-150). In this syndrome, apoptosis played a pivotal role in organ injury and failure. A dual apoptotic pathway activation in cells incubated with plasma from patients was found, as well as the production of pro-inflammatory cytokines *in vitro* (137, 148).

Unfortunately, in patients enrolled in the first phase of the study no clear trend in cell viability or death was identified among groups. It has been hypothesized that both the low sample size and the complexity of plasmatic fluid may be responsible of these results. Patients with acute decompensation in cirrhosis, and acute-on-chronic above all, had a molecular ensemble in plasma that included cell grow factors, drugs, pro-

and anti-inflammatory cytokines and mediators, excess of bilirubin and low levels of the main molecules scavenger such as albumin, that might interact with cells and gave conflicting signals. Furthermore, therapy for patients admitted in hospital (both patients with acute decompensation and acute-on-chronic) might represent another confounding factor that influences *in vitro* cell viability.

The *in vitro* effect of the main active drugs administered to patients with compensated and decompensated cirrhosis have been reported. Potassium canrenoate, a synthetic steroid and is used in the treatment of hypertension, induced a dose-dependent degree of DNA fragmentation and of DNA repair synthesis in primary cultures of hepatocytes from rat and human donors (151). Beta blockers are known to have favorable effects on endothelial function partly because of their capacity to reduce oxidative stress (152). Controversial results were collected for vitamin B12. A recent study showed that cytotoxicity activity in Caco-2 cells, a colon cancer cells, was lacked for vitamin B12 (153). It was also observed mitochondrial ROS accumulation and decreased mitochondrial SOD2 expression. Moreover, electron microscopy showed mitochondrial swelling (154). Vitamin K1 has been demonstrated as having antiproliferative and proapoptotic effects in colon cancer cell lines (155).

Insulin stimulates the growth and proliferation of a variety of cells in culture. Several hypotheses have been proposed, including regulation of essential metabolic processes and interaction of insulin with receptors for insulin-like growth factors. Evidence supporting these various hypotheses is reviewed (156).

Furthermore, it was demonstrated that pure albumin stimulates proximal tubular epithelial cells (PTEC) proliferation, and may have a role in homeostasis in health, as

well as in disrupted PTEC turnover in proteinuric nephropathies (157). Closely related results were obtained in our study when adding physiological concentration of albumin to the cell medium with plasma from cirrhotic patients, it was found a decrease in cell mortality respect the condition of low concentration of albumin.

The lacking of activation of inflammasome in cells stimulated with plasma from patients was confirmed by gene expression. The same analysis was made in PBMC of patients and it was found an interesting expression pathway of TLR-2, that increase in PBMC of patients with acute decompensation and decrease in patients with acute-on-chronic liver failure, respect a condition of compensated cirrhosis. Toll-like receptor (TLR) 2 was critically involved in production of TNF-α in response to Gram-positive microbial stimuli (158). Riondan et al. (159) showed an up-regulation of TLR-2 expression in CD14+ PBMC of cirrhotic patients, despite none of whom showed evidence of overt infection. TLR-2 expression was increase irrespective of the etiology of cirrhosis and thus is likely related to cirrhosis *per se.* Following *in vitro* PBMC stimulation with an endotoxin of S. aureus, the production of TNF-α by was inversely related to PBMC expression of TLR-2 and was significantly blunted in cirrhotic patients compared with control subject. Riondan (159) concluded saying that, even in the absence of clinically apparent sepsis, PBMC of cirrhotic patients had been presensitized by exposure to Gram-positive bacteria antigens *in vivo* and developed tolerance to such antigens *in vitro*.

The trouble of studying molecular signaling vehicled from the whole plasma led us to focus on one of its components, the EVs. These vesicles were one of the main actors responsible of cell-to-cell communication in the organism. In large scale, this communication led to both physiological and pathological crosstalk between distant

organs. The biogenesis and cellular release of the vesicles were largely described (160) and their involvement in liver disease pathogenesis was addressed through the characterization of extracellular vesicles in different scenario: nonalcoholic steatohepatitis, alcoholic hepatitis, viral hepatitis, liver fibrosis, cholangiopathies and hepatobiliary malignancies as reviewed elsewhere (160). Little is known about the characterization of EVs in the progression of liver pathology. In this project was observed that, after isolation from plasma, concentration of EVs increase with the progression of hepatopathy. Several works were published on changes in plasma levels of circulating EVs in patients with cardiovascular and other disease (69, 90, 91) or about the stimuli of EVs release relevant for liver disease (reviewed in (99) but in our case it was considered the increase in concentration related to hepatopathy stage by comparison between healthy subjects and patients and between compensated and decompensated cirrhosis, regardless of etiology of cirrhosis. About size of EVs Momen-Heravi F. (161), according to Scott et al. (Analytical Ultracentrifugation: Techniques and Methods Scott et al., 2005 The Royal Society of Chemistry, 273–276), said that EVs derived from plasma and serum had smaller sizes that reflect more Brownian motion during sedimentation (streaming factor), which could lead to reduced resolution and sedimentation efficiency.

Despite the vast majority of EVs were origined from platelets, those found in the cirrhotic patients were more frequently originated from activated endothelium. It could be hypothesized the use of a small number of markers for the characterization of platelets and monocyte origin. However, Stravitz et al. (127), in the characterization of EVs phenotypes in plasma of patients with acute liver failure and acute liver injury, reported the prevalence of platelets, hepatocyte, monocyte, but also endothelial cells,

although few in number. EVs isolated from patients expressed high levels of integrins and selectins molecules compare to healthy subjects. Surface-exposed receptors and ligands were responsible for biodistribution, for the binding of EVs to target cells or to the extracellular matrix. Subsequently, EVs may trigger intracellular signalling pathways through a simple interaction with the surface receptors or ligands of target cells or by undergoing internalization (55).

While CD40L was classically described on the surface of activated T lymphocytes, it is also found on activated mast cells and eosinophils (162). An especially important site of CD40L expression is the activated platelet (163). Therefore, following an acute injury, infiltrating platelets and inflammatory cells can both activate a variety of local structural cells, including fibroblasts, through the CD40–CD40L system (164). This means two main things: the first, it could be hypothesized that EVs characterized in this study could have platelets phenotype, underlined by the CD40L in their surface. Secondly, Henn V. and colleagues in 1998 (163) demonstrated that CD40L on platelets induces endothelial cells to secrete chemokines and to express adhesion molecules, thereby generating signals for the recruitment and extravasation of leukocytes at the site of injury. In this case we could hypothesize that both platelets and platelets-derived EVs may activated the endothelium in cirrhotic patients. Endothelium itself could release other EVs we described above. The result was a local injury and a spread of inflammatory molecules by EVs. While EVs *in vitro* effects were studied only on renal tubular cells, the results were very interesting: EVs from patients induced apoptotic death of cells that was significantly higher in acute decompensation and acute-on-chronic liver failure than that observed in compensated cirrhotic and healthy subjects. In the same times, a conspicuous production of ROS was induced which was

maximal in cells incubated with EVs from patients with acute decompensation and acute-on-chronic liver failure. The same cells showed substantially decreased in albumin uptake, that is one of the main function of renal epithelial tubular cells. Although albumin is a large anionic protein, it is not completely retained by the glomerular filtration barrier. In order to prevent proteinuria, albumin is reabsorbed along the proximal tubules by receptor-mediated endocytosis, which involves the binding proteins megalin and cubilin. Endocytosis depends on proper vesicle acidification. Disturbance of endosomal acidification or loss of the binding proteins leads to tubular proteinuria (165). Interestingly an increase in albuminuria has been found in patients with cirrhosis and acute-on-chronic liver failure (166). Christensen et al. (167) claimed that megalin, a 517-kDa monomeric protein in the proximal tubular brush border, was responsible for albumin endocytosis, according to their functional data. For this reason, also expression of megalin was investigated and it was shown that its expression did not change between the group of cells stimulated. Probably, EVs did not have effects on megalin production. EVs might have a role as inhibitory effector for albumin endocytosis but further experiments are needed. Mitochondria were essential in cells, especially if cells had active transport mechanism, such as Na+/K+ ATPase pump. In renal tubular cells stimulated with extracellular vesicles, was detected the concentration of PGC1α, a molecules involved in mitochondria biogenesis (168). The lacking of differences in PGC1α expression between the groups of cells treated suggested that EVs had no influences in mitochondria biogenesis.

The zona occludens proteins (ZO) are a family of tight junction associated proteins that function as cross-linkers, anchoring the tight junction strand proteins to the actin-based cytoskeleton (169). ZO-1 is a cell-intrinsic determinant of epithelial polarization

and lumen formation through coordination of multiple cellular processes, including mitotic spindle orientation (170). Physiological tubular handling of electrolytes is based on the maintenance of cell polarity and on the integrity of tight junction protein expression. After stimulation of tubular cells with healthy and cirrhotic groups EVs, it was observed a marked decrease of ZO-1 expression, especially between healthy subjects and patients with acute decompensation in cirrhosis. Other experiments are needed for confirm these data, but one may argue that these functional changes could alter the ability of tubular cells in maintaining compositionally distinct fluid-filled compartments with precise electrolyte concentrations.

Increased permeability of the gut has been demonstrated in patients with inflammatory disorders such as cirrhosis (171) as well as animal models of intestinal inflammation and infection (172). Alterations in tight junction architecture are one cause of increased permeability (173). Changes in the expression of these different tight junction proteins alter the resistance of the intestinal barrier. Bacterial translocation commonly occurs in both cirrhotic rats with ascites (45–78%) and cirrhotic patients (174). In our study, only renal epithelial cell line were used and a decreased expression of ZO-1 was shown in renal tubular cells after EVs stimulation of cells. However, if we hypothesize a decrease expression of ZO-1 also in intestinal epithelium after EVs actions, we could consider that EVs may be involved in increasing intestinal permeability and bacterial translocation in cirrhotic patients.

## Conclusions

In conclusion, this study showed that characterization of inflammatory profile of patients with compensated, but especially decompensated cirrhosis, need a big sample size and well selected patients for avoid confounding factors and reach statistical significance. Sometimes, cytotoxic effect of plasma is not easy to detect if we consider the whole plasma. EVs may have active role in cirrhosis: they are able to induce apoptosis and increase ROS production in renal tubular cells. Furthermore, this study demonstrates that EVs from patients with decompensated cirrhosis and acute-on-chronic liver failure are able to produce functional alteration of tubular epithelial cells such as the loss of cell polarity. We need to investigate the role of EVs in decompensated cirrhosis and study their hypothetic role as vehicle of mediator of extrahepatic organ injury and complications of cirrhosis.

## Acknowledgements

In questa ultima sessione del mio lavoro di tesi vorrei ringraziare tutte le persone che hanno permesso la sua stesura e, in generale, tutto il lavoro che ha comportato. Ma vorrei fosse anche l'occasione per ricordare chi mi ha spinto a intraprendere questa strada e mi ha permesso di raggiungere questo obiettivo.

In primis vorrei ringraziare il Professor Paolo Angeli per avermi accolto nel suo gruppo di lavoro ormai tre anni fa e avermi permesso di conoscere il suo modo di fare ricerca di altissimo livello. Tuttavia, dietro un grande mentore, c'è sempre un grande gruppo dove, personalmente, ho trovato un validissimo sostegno: compagna di avventure/sventure ma anche amica Antonietta Sticca, ormai ex-collega ma prezioso sostegno Elisabetta Gola, instancabile e grandissimo lavoratore Salvatore Piano, ma anche Antonietta Romano, Marialuisa Stanco, Filippo Morando, Marta Tonon, Elia Vettore, Chiara Pilutti. I colleghi con cui ho condiviso tutto tranne il capo sono stati altrettanto importanti per me: Cristian Turato in primis perchè con grande pazienza mi ha ascoltato e consigliato, poi Santina Quarta, Marco Villano, Mariagrazia Ruvoletto, Andrea Cappon, Alessandra Biasiolo, Brasilina Carrocia, Mariella Piazza, Giulio Ceolotto, Elisa Bertacco, Cristina Marescotti, Elisabetta Faggin e Iori, Marianna Beggio, Verdiana Ravarotto, Paul e Maria.

Ho sempre pensato di avere due famiglie, lavorativamente parlando. La seconda è capitanata dal Professor Claudio Ronco del Dipartimento di Nefrologia di Vicenza, che ringrazio in modo speciale per avermi accolta ormai più di quattro anni fa e non avermi più lasciata. Nel periodo che ho trascorso all'International Renal Research Institute di Vicenza (IRRIV) ho fatto per la prima volta "ricerca clinica", nel vero senso della parola, e ho capito che poteva essere la mia strada. Ma anche questo è stato merito di chi ho

avuto accanto: Grazia Maria Virzì, con la sua tenacia e determinazione, Massimo de Cal, un sostegno fondamentale, Anna Lorenzin, Anna Giuliani, Carlotta Caprara, Mauro Neri, Anna Clementi, Elisa Scalzotto, Francesco Garzotto, Valentina Corradi, Marco Sartori e Fiorenza Ferrari. Non posso però non ricordare le persone che ho incontrato all'IRRIV nel loro veloce passaggio e che mi hanno profondamente segnato: Faeq, Kim, Sara, Jimena, Lilia, Aashish, Akash, Percia, Debora in particolar modo.

Nel mio passato di laureanda ho incontrato fortunatamente delle persone speciali che, anche se non sento frequentemente e in alcuni casi mai, porto nel cuore: il Professor Luigi Bubacco, Nicoletta Plotegher e Stefania Girotto.

Le collaborazioni lavorative a volte arricchiscono, non solo scientificamente ma anche umanamente: per questo motivo voglio ringraziare profondamente il Professor Vincenzo Cantaluppi e Davide Medica.

Gli amici, si sa, sono sempre pronti a tirarti su il morale! Quindi ringrazio Sara Vianello, Chiara Borga e Matteo, Elisabetta Pasqualotto e Cristian, Silvia Pastori, Renata e Paolo, Marianna e Andrea, Mirco, Erica, Laura e Patrick.

Le persone più importanti in assoluto si lasciano sempre alla fine: quindi ringrazio molto volentieri la mia famiglia, soprattutto la mamma che non ha mai smesso di incoraggiarmi, di avere ansia per me nei momenti topici, di dispiacersi più di me nei momenti tristi. Però ringrazio anche papà, Filippo ed Elisa, Alberto Giada e Noeami, Andrea e Davide, anche se alcuni di loro non hanno ancora ben chiaro se ho iniziato a lavorare o no. Anche le nonne sono state importanti nel mio percorso e le ringrazio per la vicinanza.

Manuele merita un paragrafo a parte perchè soprattutto negli ultimi 4 anni ha subito tutti i miei sbalzi d'umore, pur gioendo infinitamente per i miei piccoli successi. Grazie!

Ringrazio anche Claudia e Lino per il sostegno sottoforma di ottimi momenti di convivio.

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