Post-transcriptional changes in serum albumin: role in the pathogenesis of bacterial infections in cirrhosis

Presentata da: Dott.ssa Maristella Laggetta

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ABSTRACT

Besides the oncotic function Human serum Albumin (HSA) is also endowed with many other non-oncotic properties among which the antioxidant activity. Beside quantitative changes, during cirrhosis extensive post-transcriptional alterations, likely promoted by pro-inflammatory and pro-oxidant state occur to the HSA molecule. In this study we evaluated the structural and functional integrity of HSA in a large series of hospitalized patients with advanced cirrhosis. We also evaluated the relationship between alterations to the HSA molecule and clinical features as well as 1-year prognosis of patients included in the study.

By using an LC-ESI-MS approach we identified several HSA isoforms characterized by one or more structural defects. These alterations mainly involved the cysteine-34 residue, the main antioxidant site of the molecule, and were mainly promoted by a pro-oxidant environment. Specific patterns of molecular alterations were found associated to the severity of cirrhosis and to the presence of clinical complication of disease, while the residual amount of the native HSA emerged as a potent predictor of 1-year mortality.

The functional integrity of the N-terminal portion of the HSA molecule, provided with an indirect antioxidant activity, was evaluated by measuring the circulating level of Ischemia Modified Albumin (IMA) and IMA to serum albumin ratio (IMAr). IMA and IMAr were not associated to the severity of cirrhosis nor to the patients prognosis. Contrariwise IMA and IMAr were specifically associated to bacterial infection, showing a discriminating performance comparable to that of C-reactive protein.

In conclusion this study provided evidences of clinical and prognostic relevance of HSA structural and functional alteration in patients with cirrhosis, strengthening the concept that the global function of the HSA molecule, resulting from both oncotic and non-oncotic properties, is related not only to its absolute circulating level, but also, and perhaps mainly, to its structural and functional integrity.
LIST OF ABBREVIATIONS

HSA: human serum albumin;
HSA-L: C-terminal truncated (-Leu) human serum albumin;
HSA-DA: N-terminal truncated (-Asp-Ala) human serum albumin;
HSA+SO$_2$H: sulfinylated human serum albumin;
HSA+CYS: cysteinylationed human serum albumin;
HSA+GLYC: glycosylated human serum albumin;
HSA+ CYS+GLYC: cysteinylationed and glycosylated human serum albumin;
dHSA-DA: dimeric N-terminal truncated (-Asp-Ala) human serum albumin;
dHSA-L: dimeric C-terminal truncated (-Leu) human serum albumin;
dHSA: dimeric native form of human serum albumin;
IMA: ischemia modified albumin;
IMAr: ischemia modified albumin ratio;
ESI-MS: electrospray ionization mass spectrometry;
HPLC: high performance liquid chromatography;
LC-MS: liquid chromatography-mass spectrometry;
PH: portal hypertension;
BT: bacterial translocation;
MELD: Model for End stage Liver Disease.
1. The clinical scenario: liver cirrhosis

Liver cirrhosis is the consequence of a chronic liver injury due to different etiological agents, among which hepatic viruses and alcohol are the most frequent. It is characterized by a severe tissue fibrosis that leads to a conversion of normal liver architecture leading to the formation of structurally abnormal regenerative nodules. Cirrhosis is an increasing cause of morbidity and mortality in developed countries. It is the 14th most common cause of death in adults worldwide but the fourth in central Europe; it results in 1.03 million deaths per year worldwide, 170,000 per year in Europe. In most of cases the only solution for the patients affected by cirrhosis is liver transplantation, and in Europe this pathology is the main indication for 5500 transplants per year. Different are the causes of liver cirrhosis and they vary in different area of the world. Alcoholic liver disease and hepatitis C are the most common causes in developed countries, whereas hepatitis B is the prevailing cause in most parts of Asia and sub-Saharan Africa. Liver cirrhosis could be also a consequence of genetic disorder such as haemochromatosis or Wilson’s disease. In some cases cirrhosis develops in absence of a clearly defined etiological agent, this was defined as cryptogenic cirrhosis. Additional risk factors for the progression of the disease were metabolic disorders such as type 2 diabetes, hypertension and hyperlipidemia (all features of the metabolic syndrome). They all contribute to the progression of non-alcoholic steatohepatitis leading finally to the development of cirrhosis.
1.1. Natural history of liver cirrhosis

Chronic liver injury of different etiology leads to the perpetuation of the normal wound healing response which ultimately causes an abnormal continuation of the fibrogenic process. In this setting the onset of regenerative nodules surrounded by fibrous band represents the main pathological feature based on which histological diagnosis of cirrhosis is made.

Portal hypertension (PH) represents the earliest consequence of cirrhosis and underlies most of the clinical complications of the disease. PH results from an increased intrahepatic resistance due to architectural distortion, endothelial dysfunction, intrahepatic vascular shunts between afferent and efferent vessels of the liver, and furthermore to an increase in portal blood flow.

Endothelial dysfunction is due to inhibitors of nitric oxide synthase and an increased production of vasoconstrictors that further cause a rise of hepatic resistance to blood flow and portal pressure. An increase of portal venous inflow is the result of a marked arteriolar vasodilatation in the splanchnic region draining into the portal vein. Splanchnic vasodilatation (SV) is a consequence of PH and principally related to an abnormal production of vasodilator substances such as nitric oxide, carbon monoxide, glucagon, vasodilator peptides, endocannabinoids, etc. This event leads to the attraction of blood towards the splanchnic vascular bed causing a reduced effective arterial blood volume. In this setting an enhancement of cardiac contractility and cardiac output counterbalance the vascular resistances and maintain arterial blood volume. The fall of effective arterial blood volume causes the activation of compensatory neurohormonal vasoconstrictor systems such as the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system (SNS), and arginine vasopressin. The response of these activated systems consists in retention of water, sodium and specific organ vasoconstriction, that cause the onset of typical complications which characterize the decompensate phase of cirrhosis. What explained above represents the pathophysiological mechanisms of liver cirrhosis referred to the peripheral arterial vasodilatation hypothesis (PAVH), a theory formulated in 1988, in which the splanchnic arterial vasodilatation constitutes the primary pathogenic event responsible for hypovolemia, cardiac abnormalities and finally complications such as ascites and renal impairment. Considering new findings and progress in the knowledge of liver
pathophysiology a new hypothesis has been stated\textsuperscript{10}. Such new findings attribute to the translocation of Gram negative bacteria across the intestinal wall, and to the resulting systemic inflammation, a central role in the development of vascular alteration which leads to the decompensation of cirrhosis (Figure 1). Following bacterial translocation, the bacterial product, defined as pathogen-associated molecular patterns (PAMPs), released in the vascular compartment are recognized by pattern recognition receptors (PRRs) mainly expressed on immune cells\textsuperscript{10}. The activation of PPRs leads to the production of pro-inflammatory cytokines such as interleukin 6 (IL6) and Tumor necrosis Factor α (TNFα) . The main targets for PAMPs are arterial wall and heart where they induce the production of vasodilators as nitric oxide instead for the heart they induce myocardial dysfunction\textsuperscript{10}. Thus the activation of the host innate immune response triggers endothelial molecular mechanisms responsible for arterial vasodilatation, and also promote organ damage with a storm of pro-inflammatory cytokines and reactive oxygen and nitrogen species\textsuperscript{10}.

**Figure 1.** Mechanism leading to decompensation and organ failure in patients with cirrhosis. Bacterial translocation (BT) progressively impacts the natural course of cirrhosis, from the pre-ascitic compensated stage to advanced decompensation and hepatorenal syndrome. SAV, splanchnic arterial vasodilatation. Bernardi, M. et al, *J. Hepatol.* 2015, 63:1272–84.

As outlined above the occurrence of clinical complications and the increase in portal pressure define two different phases in the natural history of cirrhosis defined as compensated and decompensated cirrhosis. The compensated phase might last for years
and is characterized from an absence of overt clinical manifestation and an hepatic vein pressure gradient (HVPG) still within a range of 5–10 mmHg. In clinical practice, HVPG represents an indirect measure of portal pressure and it is the best predictor of the development of PH.

The switch to *decompensated* cirrhosis occurs at onset of clinical complications and HVPG amounts to values more than 10–12 mmHg. Clinical manifestations of hepatic decompensation consist in ascites formation, hepatorenal syndrome, variceal bleeding, pulmonary complications and hepatic encephalopathy. Once the complication appeared the diagnosis is almost immediate, an ultrasonography or a magnetic resonance is a sufficient tool to confirm the ongoing liver cirrhosis. Instead, for the early cirrhosis, the canonical imaging could lead to false negative, so noninvasive markers of fibrosis are increasingly used like direct/indirect serum marker (AST/ALT) or new imaging modality such as Fibroscan or Fibrotest. Prediction of prognosis has a central role in the clinical management of all stages of the disease in particular for patients in severe phase. Prognostic scores based on clinical and biochemical parameters are introduced to support the clinicians in the prediction of survival and the need of liver transplantation. The most frequently used prognostic scores are the model for patients with end stage liver diseases (MELD) and the Child-Pugh scores.

The MELD score is based on creatinine, bilirubin concentrations and international normalized ratio (INR), it predicts short term survival rates (3 months) and found application in the prioritization of patients in waiting list for transplantation. The Child-Pugh score assesses the long term prognosis basing on serum level of bilirubin and albumin, INR, the presence and severity of ascites and encephalopathy and allows to classify the chronic liver disease into three risk classes (A:5-6, B: 7-9, C: 10-15).

### 1.2. Complication of liver cirrhosis

#### 1.2.1 Ascites and Hepatorenal Syndrome

Ascites is the most common complication of cirrhosis. Approximately 15% of the patients with ascites will die in one year and 44% will die in five years. Ascites formation is a direct consequence of portal hypertension and of other events characterizing the disease. In the advanced phases of cirrhosis the effort in maintenance
of arterial pressure supported by the activation of RAAS, SNS and anti-diuretic hormone (ADH) leads to retention of water and sodium. Unfortunately the prompt of fluid retention to refill dilated vascular bed is not efficient because the excessive production of lymph does not balance the return to systemic circulation and the consequence is a leakage of fluid in the abdominal cavity and formation of ascites\textsuperscript{13}. In this setting a progressive impairment of renal function occurs, leading ultimately to a peculiar type of functional renal failure defined as hepatorenal syndrome (HRS). Indeed, the activation of neurohormonal vasoconstrictor, following the reduction of the effective arterial blood volume, lead to a vasoconstriction of the kidney, brain and peripheral vascular bed. While in a first phase renal vasodilator agents, such as prostaglandins, counterbalance this effect, in later phases the system fails and is unbalanced towards a vasoconstrictor tone. Thus a reduced glomerular filtration rate (GFR) and the development of HRS is the results of a severe reduction of renal blood flow\textsuperscript{8}. HRS occurs as a late complication of advanced cirrhosis and may be triggered by infection, bleeding and paracentesis of a large volume of ascites without albumin infusion\textsuperscript{14}. The diagnosis of HRS is based on criteria provided by the International Club of Ascites (IAC). The IAC defined HRS in type 1 and type 2, type 1 rapidly progresses and it’s characterized by doubling of the serum creatinine to a level $> 2.5$ mg/dL in almost two weeks\textsuperscript{14}. The renal failure observed in type 2 HRS is moderate respect to type 1 (serum creatinine $> 1.5$ and up to $2.5$ mg/dL), the evolution of its course is quite stable and it takes on from weeks to months\textsuperscript{14}. While type 1 HRS, is normally generated by precipitating factors, HRS type 2 usually generates de novo in subjects with refractory ascites.

\textbf{1.2.2 Gastroesophageal varices}

In patients with cirrhosis, variceal bleeding is a frequent complication directly related to portal hypertension. Gastroesophageal varices are present in 30% of patients with compensated cirrhosis and 60% of patients with decompensated cirrhosis\textsuperscript{7}. The formation and increase in size of varices are driven by local anatomical factors, an increased portal pressure and progressive development of neo-angiogenesis (particularly VEGF-dependent angiogenesis) in the splanchnic territory. The development of collaterals does not achieve a reduction of portal pressure because concomitant to this
event there is an increase in portal inflow deriving from splanchnic vascular bed and the intrahepatic resistance is higher than a normal liver to be sufficiently decompressed. Indeed, collateral system with its resistance contributes to the gradient of PH and the resulted variceal wall tension is the main cause of rupture and hemorrhage\textsuperscript{15}.

1.2.3 Hepatic encephalopathy

Hepatic encephalopathy (HE) is a manifestation of decompensated cirrhosis, it occurs in 50\% of patients and it is a complication related to portal hypertension\textsuperscript{16}. The increased hepatic resistance due to the progression of liver fibrosis and development of cirrhosis forces the portal blood to bypass the liver by flowing through portosystemic shunts. The skip of the portal blood to a collateral circulation implicates a decreased first-pass effect of orally administered drugs, reduced reticuloendothelial system and detoxification function that lead to the circulation of potential dangerous metabolites. Capillarization of sinusoids and intrahepatic shunts contribute to development of HE and they cause a reduced hepatocyte perfusion, a major determinant of liver failure. HE has a wide range of clinical manifestations, from impaired memory, diminished attention to confusion and coma. Many factors are implicated in its pathogenesis as the derangements in neurotransmitter pathways, cerebral blood flow modulation, and systemic inflammatory responses. Different studies have shown that ammonia has an important role in the pathogenesis of HE. This idea is based on an impaired capacity of the cirrhotic liver to convert ammonia in urea and glutamine. Indeed, the presence of shunts towards the systemic circulation promotes a wide spread of ammonia through arterial circulation\textsuperscript{17}. Ammonia can reach the nervous central system and leads to multiple neurotoxic effects, including altering the transit of amino acids, water, and electrolytes across the neuronal membrane and propagating astrocyte swelling and cerebral edema\textsuperscript{7}. More recently, several studies suggested that increased systemic inflammation significantly contribute to the pathogenesis of hepatic encephalopathy\textsuperscript{17}. Indeed, level of serum inflammatory markers were found to be higher in patients with HE compared to those without HE regardless of the underlying severity of cirrhosis and ammonia level\textsuperscript{18}. It was postulated that bacterial translocation, together with the impaired immune defense, hepatic hemodynamic changes and porto-sistemic shunts\textsuperscript{17}, are the main driver of systemic inflammation in these patients.
The methods and biomarker selection to diagnose HE is a debating matter in particular for the identification of the Minimal Hepatic Encephalopathy, otherwise a series of specific test batteries to verify cognitive abilities have been evaluated in clinical studies but they showed to not have a good reliability and to be influenced by the population characteristic as education etc\(^9\).

The severity of HE is graded basing on the West Haven classification\(^{20}\). This scale considers 4 stages that range from manifestations as a weak loss of memory to the most severe event represented by coma and not response to painful stimuli (Table 1). Classification of patients in different stages assumes a great importance for the clinics in particular to evaluate the efficacy treatment administered.

**Table 1.** The *West-Haven classification, Z. Poh and P. E. J. Chang Int J Hepatol. 2012.*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Consciousness</th>
<th>Intellect and behavior</th>
<th>Neurological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal examination, if impaired psychomotor testing, consider MHE</td>
</tr>
<tr>
<td>1</td>
<td>Mild lack of awareness</td>
<td>Shortened attention span</td>
<td>Impaired addition or subtraction, Mild asterix or tremor</td>
</tr>
<tr>
<td>2</td>
<td>Lethargic</td>
<td>Disoriented; inappropriate behaviour</td>
<td>Obvious asterix; slurred speech</td>
</tr>
<tr>
<td>3</td>
<td>Somnolent but arousable</td>
<td>Gross disorientation; bizarre behaviour</td>
<td>Muscular rigidity and clonus; hyperreflexia</td>
</tr>
<tr>
<td>4</td>
<td>Coma</td>
<td>Coma</td>
<td>Decerebrate posturing</td>
</tr>
</tbody>
</table>

1.2.4 **Bacterial infections**

Bacterial infections are one of the leading cause of death in cirrhotic patients and mortality reported is high as 19%\(^{21}\). Prevalence of infection is mainly related to the severity of liver disease. Risk factors for these patients are previous infection, gastrointestinal bleeding, history of alcohol abuse, medical procedures and ascites\(^3\).

Cirrhotic patients are more exposed to bacterial infections compared to patients with other illnesses. First, they present alterations of bacterial flora and mucosal barrier, which concur, together with a major permeability of the latter, to a greater bacterial migration. Furthermore immunologic response is altered and partially impaired, thus favoring the progression and spread of infection\(^{22}\). Once the infection has established, it can lead to complications such as severe inflammatory response, renal failure, encephalopathy and in worst case scenario multiorgan failure\(^{22}\).
Spontaneous bacterial peritonitis (SBP) and urinary tract infections are the most frequent infections followed by pneumonia, bacteremia and finally skin and soft tissue infections. Intestinal Gram-negative flora is the major cause of infections, several evidences suggests that bacterial translocation (BT) from gastrointestinal tract is the main mechanism which favors bacterial infection. The onset of complications following BI underlines the need for vigilance and rigorous antimicrobial approach. The factors, predisposing cirrhotic patients to infections, are not well defined but several mechanisms are suggested. PH results in the formation of portosystemic anastomoses that, diverting blood from liver, determines an impairment of the hepatic detoxification function. The intra and extra-hepatic shunting of portal blood into the systemic circulation (hemodynamic derangement typical of decompensated cirrhosis) causes a progressive failure in detoxification of potentially harmful substances and consequently bacterial end-products received from the splanchnic circulation are released in the systemic circulation. As previously outlined, bacterial translocation from gastrointestinal tract contributes to the onset of infections. It is due to an enhanced intestinal permeability, which results from several factors, among which oxidative damage of the intestinal wall, alteration in tight junctions of the intestinal epithelium, compromised Paneth cells defense and altered expression of anti-microbial peptides. Depending from the etiology of cirrhosis, gut flora is subjected to changes of its own composition. These changes can lead to dysbiosis and a further overgrowth of harmful strains that increase the potential for infection. In healthy individual BT can be easily controlled by the immune system, whereas for cirrhotic patients this event could comprise the survival. The reason is because cirrhosis can lead to a dysfunction of the immune system which consists in two main alterations: immunodeficiency related to a weaken response to pathogens and a systemic inflammation, as a result of persistent and inadequate activation of immune cells. The immunodeficiency consists in an impairment of innate and adaptive immune system (Figure 2) due to a series of factors: compromised immune mechanisms of the mesenteric lymph nodes, insufficient bacteriostatic and serum chemotactic capacity, a reduced neutrophil phagocytosis, a decreased number of circulating immune system cells and an hepatic reticuloendothelial dysfunction. Importantly, a distinctive element that occurs at the same time of immunodeficiency is a systemic inflammation often
associated with marked immune activation, which correlates with the severity of liver
disease and predicts survival in these patients. This phenomenon initiates in advanced
phase of liver cirrhosis, following BT which contributes to systemic inflammation.
PAMPs are recognized by PRRs on innate immune cells. The main recognition for
bacteria and PAMPs occurs in the gut-associated lymphoid tissue (GALT) and
mesenteric lymph nodes (MLN) and in the peripheral blood. Once the immune cells are
activated in the GALT and MLN, they enter the peripheral blood and spread the
inflammatory response systemically.

Cirrhotic patients frequently present systemic inflammatory response syndrome (SIRS),
that is a complication related to the presence of a serial factors as bacterial translocation,
impaired reticuloendothelial function and portosystemic shunts. The result is an
increased level of endotoxins in the systemic circulation, and a further activation of
immune cells as monocytes that cause an excessive release of pro-inflammatory
cytokines (interleukin-1, interleukin 6, TNF-α, interferon Y). In cirrhotic patients with
SIRS, the onset of bacterial infection implicates a pronounced inflammatory response
with elevated systemic concentrations of pyrogenic cytokines as IL-6 and TNF-α.
Figure 2. Rearrangement of the hepatic and systemic immune system in liver cirrhosis. Albillos A et al, J Hepatol. 2014 Dec;61(6):1385-96.
2. Human Serum Albumin

Human serum albumin (HSA) is the more abundant plasma protein which accounts for almost 50% of total plasma protein content. The first clinical application for HSA was during the second world war, when, thanks to its property of plasma expander, it was administered to severely burned patients injured during the Pearl Harbour attack. Subsequent advance in the knowledge of HSA properties, have led to a widening of the clinical indication for the use of HSA. Indeed, HSA is provided of many other properties which are unrelated to the regulation of fluid compartmentalization and to the oncotic pressure, these properties are therefore defined as non-oncotic. HSA is synthesized by hepatocytes, parenchymal cells of the liver, through the transcription of its gene located on the long arm of chromosome 4, for an amount of 10-15 gr. per day. Once synthesized, a part is stored in tissues (like muscle and skin) instead a consistent amount is released in the vascular space. Circulating albumin leaves plasma at a rate 5%/h and returns in the vascular space at an equivalent rate through a lymphatic system. HSA total half-life in an healthy male lasts from 12.7 to 18.2 days, while its circulatory half-life is much shorter, reaching approximately 16-18 hours. HSA was synthesized following stimuli provided by hormonal factors, such as insulin, cortisol and growth hormone and changes in the plasma oncotic pressure, while pro-inflammatory mediators, including cytokines, Interleukin-6 and Tumor Necrosis Factor-α exert an inhibitory effect.

2.1 Structure

HSA is a small globular protein of 67 KDa, composed of 585 amino acids, with a negative charge (-15) at ph 7 due to an high content of acidic amino acids like lysine, aspartic acid, only one residue of tryptophan and few methionine residues. The tertiary structure of HSA, as observed in x-ray crystallographic studies, is heart shaped, and is mostly composed of alpha helices. The molecule is organized in three homologous domains (I-II-III), each composed of two subdomains (A and B) with 4 and 6 alpha helices respectively (Figure 3). Thanks to its amino acid composition, HSA has a structure enough flexible to be prone to bind several molecules, the presence of 35 cysteines contribute to tertiary structure through the formation of 17 intra-molecular disulfide bridges. Only one cysteine at 34 position (Cys-34) has a free redox active thiol group (-SH) that can undergo to thiolation, nitrosylation, and reversible or irreversible
oxidation. Thanks to the presence of a free thiol group in its structure and the high content in the bloodstream, HSA represents the main circulating anti-oxidant system.

Figure. 3 Crystal structure of human serum albumin, subdomains (IA, IB, IIA, IIB, IIIA an IIIB), of the N and C-terminal, Sudlow’s sites I and II and the seven LCFA binding sites (FA1 to FA7) are shown. Purple spheres represent the heavy atoms of side chain of residue Cys-34. Arroyo V. et al, J Hepatol. 2014 Aug.;61(2):396-407

2.2 Functions

2.2.1. Oncotic properties

HSA is the main regulator of fluid distribution in the body compartments, indeed, it is responsible for 80% of the oncotic pressure of plasma (25–33 mmHg). The osmotic pressure and the Gibbs-Donnan effect are the main mechanisms through which HSA influences the fluid distribution. Indeed, approximately two thirds of the plasma oncotic pressure is represented by a simple osmotic pressure, due to HSA high plasma concentration. The other third arises from the Gibbs-Donnan effect, namely, the molecular negative net charge (at physiological pH) attracts positively charged molecules into the intravascular compartment. Furthermore HSA binds the interstitial
matrix and sub endothelium reducing the permeability of these layers to large molecules, so that it may indirectly increase the intravascular oncotic pressure\textsuperscript{38}.

2.2.2 Non-oncotic properties

\textit{Binding and transport}. The HSA binding properties are related to its tertiary structure and multidomain organization. HSA binds molecules in order to increase their plasma solubility, to transport them to tissues and organs or to remove them when they are toxic\textsuperscript{31}. HSA can transport and bind both exogenous and endogenous molecules. An example for endogenous compound is the group of long chain fatty acids (LCFA), that can circulate free or bound to HSA. HSA has 7 sites through which binds LCFAs with different affinity, Sudlow site I e II are included (Sudlow site are respectively in the subdomain IIa e IIIa of the molecule)\textsuperscript{30}. The binding capacity of HSA is not limited only to LCFA but also to other endogenous molecules like eicosanoids, bile acids, unconjugated bilirubin, steroids, hematide, vitamin D and folates\textsuperscript{30}. Regarding the interaction with exogenous molecules, several classes of drugs bind HSA, among which benzodiazepines, anticoagulants, non-steroidal anti-inflammatory drugs and antibiotics\textsuperscript{39}. The binding of drugs to HSA is an important determinant for their efficacy and biodistribution. The main sites involved in drug interaction are Sudlow site 1 (for anticoagulants and anti-inflammatory drugs) and Sudlow site 2 (for benzodiazepines and profens) but an higher drug concentration may involve other binding sites, whereas molecules as cisplatin, D-penicillamine, and N acetyl-cysteine bind to Cys 34 residue\textsuperscript{40} (Fig. 4). The binding properties of albumin could be influenced by several physiological or pathological factors such as age, genetic factors, reversible or covalent binding of endogenous ligands and xenobiotics that may alter the functionality of the binding sites of the molecule\textsuperscript{39}. 
Figure 4. Crystal structure of human albumin showing the binding sites. The α-helical structures of the three domains (DI, DII, and DIII) are divided into subdomains (A and B) as indicated. DI (pink) contains the fatty acid binding site 1, the free cysteine (C34), and drug binding site 3. Fatty acid site 2 is located at the interface between DI and DII. The metal binding site is located between subdomain DIA and DIIA. DII (orange) contains the drug binding site 1 (Sudlow’s site 1) as well as fatty acid sites 6 and 7. DIII (blue) contains fatty acid binding sites 3 and 4, the drug binding site 2 (Sudlow’s site 2) in DIIIA, and the fatty acid biding site 5 in DIIIB. Sand KM et al. Front Immunol. 2015 Jan 26. 2015 Jan 26;5:682

Anti-oxidant function. The anti-oxidant function awarded to HSA is mainly related to the capacity to bind metal ions at the N-terminal portion of the molecule and to the redox ability of the Cys-34 residue. Free metal ions could catalyze the formation of ROS (reactive oxygen species) and lead to Fenton reaction producing hydroxyl radicals. The production of ROS has a dangerous effect on biomolecules leading to cell damage and HSA may limit this effect. Indeed, the N-terminal portion of HSA is provided by an indirect antioxidant activity. Thanks to its amino acid sequence (N-Asp-Ala-His-Lys) it can bind metal ions as Cu (II), Co (II) e Ni (II) reducing their contribute to the generation of ROS and oxidative stress. Beside the amino terminal portion of the protein, the Cys-34 residue has a direct anti-oxidant activity. The presence of a free thiol group at Cys-34 residue leads albumin to be in plasma the main source of extracellular thiols (80% ) and a potent scavenger for ROS and reactive nitrogen species.
In healthy adults about 70-80 % of HSA exists predominantly in a reduced form with a free sulphhydryl group at Cys-34 and preserved anti-oxidant ability, known as mercaptoalbumin (HMA). A small amount that consists in the 25 % of circulating protein is human nonmercaptalbumin1 (HNA1), in which Cys-34 is reversibly oxidized and it forms a disulfide bridge with small sulfhydryl groups like another cysteine, homocysteine or glutathione. A residual amount, representing approximately 5% of the total circulating HSA is human nonmercaptalbumin2 (HNA2), which is highly and irreversibly oxidized to sulphinic or sulphonic acid form leading to a permanent loss of its scavenger ability. Oxidative modifications of the albumin molecule cause not only a loss of its anti-oxidant activity but interfere with the pharmacokinetics of the molecule itself, because the oxidized forms are degraded and removed faster.

**Immunomodulation and anti-inflammatory properties.** Albumin carries out its immunomodulating function at different levels, through the regulation of neutrophil/endothelial cell interactions after shock and resuscitation, and influencing the intracellular glutathione levels (GSH) (an ubiquitous sulfhydryl thiol) which takes part into the cell protection mechanism against oxidant-mediated injury. In vitro and in vivo studies showed that albumin does not directly interact with GSH by the Cys-34 residue, but once internalized in the cellular compartment, it represents a source for precursors that lead to the de novo synthesis of GSH. The consequent effect of the increase of intracellular GSH is the downregulation of the ubiquitous transcription factor nuclear factor-kappa B (NF-kB), following of a TNF α activation (oxidant-sensitive transcription protein complex). Furthermore HSA can interact with pro-inflammatory substances and mediators of inflammation. Indeed, HSA can bind lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycans which are surface components of gram-negative and gram-positive bacteria that activate the innate immune system through the binding to Toll-like receptor 4 (TLR4) thus inducing the transcription of pro-inflammatory compounds. To activate the inflammatory response, LPS has to interact with several host proteins as LPS-binding protein, CD14 and co-receptor MD-2, leading to cell activation. Recent findings suggest that Albumin can participate to the presentation of LPS to TLR4, otherwise the resulting immune activation is moderated respect to the endotoxin-CD14 complexes. Giving that circulating albumin is abundant and LPS-albumin complex has
a moderated effect on cell activation, indeed HSA could play a role in moderating the inflammatory response to bacterial infections\(^45\). Therefore, albumin could play a dual role either stimulating or moderating immune cells activation depending on pathophysiological conditions\(^44-45\).

**Anti-hemostatic effect.** Nitric Oxide (NO) is a highly reactive molecule which causes vasodilatation in the vascular compartment\(^33\). Some studies showed that endogenous or exogenous NO interacts with Cys 34 residue of HSA through an electrophilic addition of nitrosium ion (NO\(^+\)). Thanks to this process, NO can circulate in plasma as a S-nitroso HSA adduct. The vasodilator properties of NO increase when it is transferred to low-molecular-weight thiols and consequently also its circulating availability\(^33\). S-nitrosothiol adducts with respect to a free NO have a longer half-life, they can inhibit platelets through a cGMP-dependent mechanism and they possess endothelium-derived relaxing factor-like properties\(^35\).

**Endothelial stabilization.** More than 50\% of total body albumin is located into the extravascular compartment, and some studies on hydraulic conductivity carried out on monolayers of endothelial cells showed that HSA was able to influence vascular integrity \(^46\). *In vitro* experiments demonstrated that increasing amount of HSA in luminal space causes a rise in barrier function and reduced vessel wall permeability thanks to the binding and to an increment of its number of interactions with extracellular matrix\(^46\). HSA beyond its influence on capillary permeability may also act on endothelial stabilization. This hypothesis is supported by ex-vivo studies \(^47\) and a randomized clinical trial \(^48\) on patients with spontaneous bacterial peritonitis (SBP) in which HSA administration effectively improved systemic hemodynamics and reduced endothelial dysfunction respect to other colloids thus strengthening the role of albumin as endothelial stabilizer.

3. **HSA in liver cirrhosis**

HSA is exclusively produced in the liver, therefore during chronic liver disease its circulating concentration is reduced leading to a condition of hypoalbuminemia\(^49\). A reduced concentration of HSA results in part from a decreased synthesis related to
parenchymal cells loss and in part from vascular abnormalities which occurs during cirrhosis. Namely, the retention of renal sodium and water following splanchnic arterial vasodilatation, lead to plasma volume expansion and to a further dilution of the extracellular fluid protein content including that of HSA. In the more severe phases of disease the increase in the trans-capillary escape rate of HSA further contributes to the reduction of its circulating level. Thus, in the field of hepatology, the administration of commercial HSA preparations is now indicated for the treatment or prevention of severe complications of cirrhosis. In lights of new findings about HSA properties, benefits after its administration have been ascribable not only to its plasma volume expander ability but also to its non-oncotic properties.

3.1 Clinical indications for the use of HSA in patients with cirrhosis

Clinical use of HSA for cirrhosis dates back to the forties years, when the main indication for albumin infusion was ascites because there was the idea that hypoalbuminemia could be the main cause for ascites formation. In the years between 1940-1960 several studies were carried out to evaluate the effects of HSA administration on the management of ascites. In these studies the administration of HSA was aimed to reduce ascites formation by increasing microvascular oncotic pressure and stabilizing circulatory and renal function through the expansion of total blood volume. The observed results from these studies showed an increase of serum albumin levels and normalization of the oncotic pressure, however the treatment did not lead to a considerable reduction in ascites formation, even if the therapy lasted for prolonged periods. Although these studies failed to reduce ascites formation, they demonstrated that a reduction in oncotic pressure did not play a role in the pathogenesis of ascites formation.

Actually the clinical indications for albumin administration in patients with cirrhosis are the prevention of circulatory dysfunction induced by paracentesis of large volume of ascites, the treatment of HRS and prevention of renal complications due to spontaneous bacterial peritonitis. Otherwise, clinical studies are currently evaluating the appropriateness of the use of HSA for the improvement of patients prognosis and the treatment or prevention of other complications as bacterial infections, hepatic encephalopathy and septic shock.
3.1.1 Prevention of post-paracentesis circulatory dysfunction

The progresses in the knowledge of liver cirrhosis physiopathology showed that cardiovascular abnormalities were involved more than hypoalbuminemia in the development of ascites. Therefore, in the eighty years, commercial HSA preparations found application in patients undergone paracentesis of large volume (LVP, more than 5L) of ascites to prevent post-paracentesis circulatory dysfunction (PPCD). This nosocomial procedure represents the first line treatment for refractory ascites and consists in the removal of large amount of ascitic fluid from the peritoneal cavity.

LVP may lead to circulatory dysfunction (CD), deriving from the activation of the renin-angiotensin system which contributes to an exacerbation of the arteriolar vasodilatation and an insufficient cardiac response. Normally CD does not revert spontaneously and is associated to an increased risk of mortality and acute renal failure. Albumin infusion given at the same time of LVP or at the end of the procedure has shown to be effective in the prevention of CD. The reason of this positive effect consists in the ability of albumin to be a plasma volume expander with a longer persistence in the vascular compartment (21 days of half-life in cirrhotic patients) respect to other artificial colloids. Its prolonged half-life favors the maintenance of circulatory homeostasis, that is severely compromised in these patients. Clinical trials further confirmed that albumin administration compared to other fluid volume expanders is able to reduce the development of complications related to PPCD as hyponatremia, renal failure and risk of mortality. A better performance of HSA respect to other known colloids lets to hypothesize the beneficial effects of HSA extend beyond its oncotic function.

3.1.2 Treatment of Hepatorenal syndrome

Hepatorenal syndrome (HRS), as explained previously, is a severe and progressive functional renal failure occurring in patients with cirrhosis and ascites characterized by splanchnic arterial vasodilatation in association with cardiac dysfunction. A worsening of circulatory dysfunction coupled to activation of compensatory mechanisms as RAAS and sympathetic nervous system cause renal hypoperfusion and organ dysfunction. Even if the HRS are distinguished in type 1 and type 2, the main investigations and collected information regard type 1 HRS. Recent data showed that the combination albumin-vasoconstrictors resulted the most effective treatment with respect to the use of
vasoconstrictors alone. The reason of the effectiveness of this combination resides in the fact that albumin increases renal blood flow, glomerular filtration rate and decrease serum creatinine level, on the other hand vasoconstrictors act at the splanchnic vascular bed level increasing vascular resistances and consequently the mean arterial pressure and renal perfusion. The most studied vasoconstrictor is terlipressin, that, administered in association with HSA, is effective in the improvement of renal function and in the full reversion of type 1 HRS in 40-50% of patients. Also in decompensated cirrhotic patients affected by acute kidney injury (AKI) albumin infusions showed to improve renal blood flow. The assessment in these patients of markers related to endothelial dysfunction, oxidative stress and endotoxemia revealed that the beneficial effect of HSA infusion is mainly related to the endothelial stabilization and to the reduction of oxidative stress more than to its oncotic function.

3.1.3 Prevention of renal complications related to SBP

Patients affected by SBP show an increased level of cytokines and vasodilator factors in plasma and ascitic fluid. These compounds lead to changes in effective arterial blood volume that further contribute to the onset of renal complications. HSA administration achieved an improvement of hemodynamics in patients with SBP, this effect was showed to be not only related to oncotic function but also to the immunomodulation, antioxidant and endothelial stabilization capacity of the protein. A clinical trial, in which the effect of HSA infusion was compared with that of another colloid, demonstrated a rise of systemic vascular resistances after HSA administration, and a fall of the circulating levels of von Willebrand-related antigen, factor VIII, and nitric oxide metabolites, indicating that HSA can interfere with the endothelial activation. Furthermore, other clinical studies showed that the combination of HSA and antibiotics in patients with SBP could better reduce the incidence of renal failure and the mortality rate. The beneficial additional effect of HSA administration to the antimicrobial therapy was supported by another trial where the combination of cefotaxime and HSA with respect to the antibiotic alone resulted in an almost avoided occurrence of renal impairment and lowered mortality both during hospitalization and after 3-months from the discharge. Therefore, the achieved benefits after HSA administration clearly appear to be not only related to its oncotic function but also to many other physiological non oncotic functions.
4. Post-transcriptional modifications of HSA during cirrhosis

Pathological conditions as sepsis, diabetes, chronic renal failure and cancer are characterized by production or accumulation of endogenous ligands or substances that can bind and/or oxidize HSA leading to changes in its structure and functionalities. Liver cirrhosis takes part to the list of disease able to induce these alterations\textsuperscript{32}. Indeed, several studies, through the analysis of plasma HSA from cirrhotic patients, confirmed the idea that liver cirrhosis could induce changes in albumin non-oncotic functions. Watanabe \textit{et al} \textsuperscript{65} showed that the percentage of oxidized albumin increased with the progression of cirrhosis. Namely, oxidized albumin was found to progressively increase over the three different Child Pugh classes in parallel to a reduction of the total serum albumin concentration (Figure 5)\textsuperscript{65}.

![Figure 5](image-url)

**Figure 5.** Oxidized albumin percentage (black bars) and serum albumin level (white bars) measured through an immunoassay in healthy controls and cirrhotic patients. Watanabe \textit{et al} Nutrition. 2004 Apr.;20(4):351-7.
In the same study an increased percentage of glycosylated albumin in cirrhotic patients was also demonstrated. Glycation is another post-transcriptional modification that occurs through a non enzymatic reaction and involves glucose and/or other sugars that attach free amino group of albumin. This modification may be somewhat expected in patients with liver cirrhosis because one third of patients suffer of diabetes.

A further characterization of oxidative modification to the HSA molecule during cirrhosis was performed by Oettl et al. The authors observed that in cirrhotic patients there is a significant trend towards the reversible and irreversible oxidation of the Cys-34 residue of the HSA molecule as revealed by the plasma HMA, HNA1 and HNA2 levels. Such result was confirmed in subsequent studies performed in larger cohort of cirrhotic patients in which an higher plasma HNA2 level was observed in decompensated cirrhosis. In the same study the HNA2 level significantly correlated with the severity of cirrhosis and was a good predictor of short and medium term mortality.

Beside oxidative modifications at the Cys-34 level, post-transcriptional changes can occur also in other sites of the HSA molecule. In the early 1990, Ischemia modified albumin (IMA) was described for the first time. At the beginning, this protein variant seemed to be produced only under myocardial ischemia condition, thereafter several studies assessed its presence in other pathological conditions as liver cirrhosis, acute infection and advanced cancer. The N-terminal portion of HSA displays an indirect anti-oxidant activity being able to bind metal ions. Otherwise the site is susceptible to biochemical degradation and is less stable under environmental changes, so it could be affected by hypoxia, acidosis and free radicals. Indeed IMA consists in an isoform with altered amino terminal site which causes a transient loss of scavenging ability leading metals to circulate free and participate into reactions that produce detrimental metabolites for patients.

Jalan et al. have measured plasmatic levels of IMA in patients with different grades of cirrhosis severity. In this study the authors found that the ratio IMA/serum albumin concentration (IMAR) increased with the severity of cirrhosis, a result that mirrored the increase of oxidative stress which characterizes the more advanced phases of the disease. IMAR was also found to be an eligible biomarker to predict mortality and to determine survival in patients with acute decompensation of cirrhosis.
During the last ten years, new proteomic techniques as High-performance liquid chromatography (HPLC) coupled to mass spectrometry (ESI-TOF MS) allowed the better identification and characterization of HSA structural alterations. This method was initially used from Bar-Or and colleagues to evaluate post-transcriptional changes of HSA in different commercial preparations of albumin and to compare them to that found in circulating HSA from healthy volunteers. Cysteinylation and nitrosylation of the cysteine 34 residue were described together with a S-nitrosoalbumin isoform. It’s well known that NO bound to albumin has a prolonged half-life moreover experimental studies conferred to this isoform anti-inflammatory action as in case of lung acute injury due to endotoxemia and a protective effect on liver cells in animal models of ischemia/reperfusion injury. Otherwise, nitrosylation of cys34 causes a decrease in allosterical affinity to copper ions and to other molecules. An additional modifications described by Bar-or and colleagues was the truncation at the N-terminal portion of HSA. This change consists in a loss of the last two aminoacidic residue (Asp-Ala) from the N-terminal portion of HSA, the site of the molecule involved in the binding of transition metals ions. The truncation and further loss of this site means a permanent loss of HSA scavenging ability.

More recently, our group revisited the method initially described by Bar-Or and colleagues in order to render this approach reliable to be applied in clinics for the assessment of circulating albumin microheterogeneity. With this approach several HSA isoforms were identified in plasma samples from healthy voluntaries and from a small series of cirrhotic patients. Namely, isoforms characterized by reversible (HSA+CYS) or irreversible (HSA+SO$_2$H) oxidation of the Cys-34 residue, truncation of the N-terminal (HSA-DA) and C-terminal (HSA-L) portion of the molecule, glycosylation (HSA+GLYC), and the co-occurrence of such structural alterations (HSA+CYS-DA: N-terminal truncated and reversibly oxidized; HSA+CYS+GLYC: reversibly oxidized and glycosylated) have been identified (Figure 6).
These results were congruent with those obtained from Bar-or\textsuperscript{74} and the method was tested for inter- and intra-day reproducibility. The validation of this method gave the opportunity to extend the analysis of albumin structural alterations in patients in order to provide additional information about the structural integrity of circulating HSA. In this view the evaluation of HSA structural alteration in a large cohort of cirrhotic patients will provide the opportunity to match clinical observations to information about albumin microheterogeneity thus unraveling the potential clinical relevance of such structural alterations.
4.1 Other post-transcriptional changes of HSA

Additional structural modifications of the HSA molecule were described in clinical settings other than cirrhosis. Indeed, the formation of albumin homodimers has been proposed as an oxidative structural modification that occurs to the HSA molecule in-vivo$^{77}$. The formation of albumin disulfide dimers was already reported in dialyzed human plasma following exposure to peroxynitrite, additionally the concentration of albumin dimers was significantly increased in the circulating blood of patients with chronic renal disease, compared to age-matched healthy subjects $^{77}$ (figure 7). The dimerization process seems to involve a disulfide bonds at the Cys-34 residue thus contributing to a further reduction of the non-oncotic activity of the circulating albumin pool by impairing the antioxidant properties of the protein$^{78}$.

![Figure 7](image)

**Figure 7.** Detection of albumin dimer in plasma from healthy subjects (lane 1-3) and hemodialysed patients (4-6) by western blot. Monomeric albumin and albumin dimers are indicated by black arrows. Formation of albumin dimers induced by exposure to peroxides in human plasma: a possible biomarker for oxidative stress. Ogasawara et al, K. Biochem. Biophys. Res. Commun, 2006. 340, 353–8.
AIMS OF THE STUDY

The main aim of this project was studying in a large cohort of cirrhotic patients, admitted to the hepatological units of Sant’Orsola-Malpighi Hospital (Bologna, Italy) for the onset of a complication of the disease, the functional and structural alterations of Human serum albumin.

First, by using an innovative technological approach based on mass spectrometry we will identify and characterize, structural modifications to the HSA and the sites of the molecule involved. Once the number of different existing isoforms and the extension of alterations will be assessed, we will analyze their association with clinical complications and severity of the disease and, if any, their prognostic role.

Basing on the hypothesis that oxidative stress, as it develops during cirrhosis, could be the main driver of HSA structural alteration, further experiment will be performed in vitro to assess the relationship between a pro-oxidant environment and modifications of albumin structure.

An additional aim of the study is the evaluation of functional alterations of HSA during cirrhosis by analyzing the N-terminal portion of the molecule, which carries an indirect antioxidant activity, in the same cohort of patients. Then we will evaluate if such alteration is related to the severity of disease and/or associated to specific complications. Finally, additional evaluations will be performed in rats with experimental cirrhosis in order to further characterize the relationship between acute event, such as bacterial infection, and the functional alteration of the N-terminal portion of the albumin molecule.
MATERIALS AND METHODS

1. Patients
From July 2011 to March 2012, all patients admitted to the hepatological units of the S. Orsola-Malpighi university hospital for the onset of a clinical complication of cirrhosis were screened for study enrollment. Exclusion criteria were age: under 18 years, admission for a scheduled procedure, hepatocellular carcinoma (HCC) exceeding the Milan criteria, heart and respiratory failure, organic renal diseases, onco-hematologic disorders, protein-losing syndromes, albumin infusion in the previous month, and ongoing immunosuppressive treatment. A group of patients with a stable cirrhosis attending the outpatient clinics for scheduled surveillance and age and sex comparable healthy voluntaries to be used as reference population were also evaluated. Informed written consent was provided by patients and healthy controls, and the study protocol approved by the local ethical committee according to the 1975 Declaration of Helsinki.

2. Study design and definition
At the time of the inclusion in the study for each subject peripheral blood sample was withdrawn from the brachial vein into pyrogen-free tubes (Vacutainer EDTA and Heparin tubes; Becton Dickinson Italia, Milan, Italy), and immediately centrifuged at 3,000g for 10 minutes. Plasma samples were than aliquoted into cryotubes (Corning Inc., Corning BV, Amsterdam, The Netherlands) and stored at -80°C until analysis. For both hospitalized patients and outpatients, a routinely biochemical evaluation, including tests of liver and renal function, and coagulation parameters were performed. Prognostic scores as Model of End-stage Liver Disease (MELD)\(^79\), Child-Pugh\(^80\) and Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA)\(^81\) were calculated when appropriate to assess the disease severity. The diagnosis of cirrhosis was based on clinical, biochemical, ultrasound (US) or endoscopic features. Evaluation of complication was performed as follows: ascites through ultrasound examination; hepatic encephalopathy was graded according to the West-Haven classification\(^82\); renal impairment was defined as a serum creatinine greater than 1.5
mg/dL; and upper gastrointestinal bleeding was confirmed by endoscopy. Diabetes was diagnosed according to the American Diabetes Association guidelines.

The occurrence of Bacterial infection was evaluated according to the following criteria:

- spontaneous bacterial peritonitis: polymorphonuclear cell count in ascites >250/mm³;
- urinary infection: positive urine culture or >10 leukocytes per high-power field in urine associated with suggestive clinical symptoms and signs of bacterial infection;
- pneumonia: presence of new infiltrates on chest X-ray;
- skin/soft tissue infection: physical examination findings among which swelling, erythema, heat and tenderness of the skin;
- spontaneous bacteremia: positive blood cultures and no evident cause of bacteremia;
- other infections (diverticulitis, cholecystitis, and meningoencephalitis): according to specific findings at the laboratory, microbiological, and imaging assessment.

Hospitalized patients were followed-up until discharge and up to one year for survival.

3. Evaluation of HSA structural alterations

3.1 Identification and relative quantification of HSA monomeric isoforms

3.1.1 Sample preparation

EDTA-treated serum samples were diluted 1:10 with water/acetonitrile (98/2; v/v) mixture and filtered with an 0.22-µm filter (Merck KGaA, Darmstadt, Germany). A further dilution (1:100) with the same mixture was then performed before each injection in LC-MS system. In order to obtain a certain identification of HSA isoforms, plasma sample from CH patient containing approximately 95 µg of HSA was treated with DTT 10 mM in ammonium bicarbonate buffer (100 mM, pH 8.0) for 30 min at 56°C to reduce disulfides. After cooling, sample was treated with iodoacetamide 55 mM in ammonium bicarbonate buffer (100 mM, pH 8.0) at room temperature for 45 min in the
dark to alkylate cysteine residues. The plasma sample was finally digested using trypsin overnight at 37º.

In a different experiment setting, focused on the identification of Cys34 alterations, serum sample containing approximately 3.5 µg of HSA was directly digested overnight with trypsin. All the tryptic digested were analysed by an LC-ESI/MSMS approach.

3.1.2 HSA LC/ESI-MS analysis

HSA LC-MS characterization was performed adopting a validated and optimized protocol previously published from our group. A nano-LC (liquid chromatography)/nano-ESI/Q-TOF (quadruple time-of-flight) analysis was performed by using a nano-LC Agilent 1100 Series (Agilent,Walbronn, Germany) interfaced with a Q-TOF hybrid analyzer (Q-TOF Micro; Micromass, Manchester, UK) with a nano-Z-spray ion source. Reversed-phase chromatographic separations of HSA from other serum proteins was performed on a C8 (50 mm x 75 µm; 3.5 µm) column, using an elution gradient from A (water: acetonitrile: FA [99:1:0.1] [v/v/v] / B (acetonitrile: water: FA [98:2:0.1] [v/v/v] 85/15 [v/v]) to A/B 20/80 (v/v), in 20 minutes, at a flow rate of 0.5 µL/min; the system was equipped with an auto-sampler, and the injection volume was 1 µL. The column was equilibrated with the mobile-phase composition of the starting conditions for 10 minutes before the next injection. The mass spectrometric analysis was performed by setting the source temperature at 100 ºC, the capillary voltage at 3.5 kV, and the cone voltage at 42V. The scan time was set at 2.4 seconds and the interscan time at 0.1 sec. Mass chromatograms were recorded in total ion current (TIC) mode in the mass range 600-2,500 m/z (mass/charge). To characterize HSA isoforms by molecular-weight determination, multicharged mass spectrum was acquired on the chromatographic peak apex identified as HSA. Deconvoluted ESI mass spectrum of HSA was obtained by using MassLynx 4.1 software (Waters Corporation, Milford, MA). The peak averaged mass spectra were reconstructed and the mass of the HSA isoforms derived.

For each isoform the absolute intensity was computed and then the relative abundance of each isoform was determined according to the following formula:
3.1.3 HSA isoforms identification by 2D-LC-ESI-MSMS analyses

The first dimension RP-LC separation was performed on Agilent 1200 HPLC System (Walbronn, Germany) by using a Zorbax Extend-C18 RP column (3.5 µm, 80 Å, 100 mm × 2.1 mm i.d.). Mobile phases A [water/acetonitrile (98/2) (v/v), adjusted pH to 10.0 using ammonium hydroxide] and B [acetonitrile/water (98/2) (v/v), adjusted pH to 10.0 using ammonium hydroxide] were used to develop a gradient from 2 to 70% of mobile phase B in 20 min. Tryptic peptides (~30 µg) were separated at an eluent flow rate of 0.3 mL/min and monitored at 214 nm. Eluent was collected every 2 minutes. The samples were dried under vacuum and reconstituted in 30 µL of 0.1% (v/v) FA, 2% (v/v) acetonitrile in water for subsequent analyses. Second dimension separation was carried out by using a nano-LC Agilent 1100 Series (Walbronn, Germany). Analyses were performed on a C18 (150 mm x 75 µm; 3.5 µm) column. Mobile phases A [water: acetonitrile: FA (99:1:0.1) (v/v)] and B [acetonitrile: water: FA (98:2:0.1) (v/v)] were used to develop a gradient from 5 to 55% of mobile phase B in 70 min.

Mass spectrometry analyses were performed on the Q-TOF (Micromass, Manchester, UK) with nano-Z-spray ion source. The ESI-QToF source temperature was set at 110°C, the capillary voltage at 3.5 kV, and the cone voltage at 40 V.

Fragment ion spectra obtained from LC-ESI-MS/MS analyses were processed using Mascot Distiller (Matrix Science, London, UK) and the data were analyzed by searching the human SWISSPROT database (www.uniprot.org). Oxidation to sulfonic and sulfinic acid, nitrosylation and cysteinylation at Cys (amino acid) residues, glycosylation at Ser, Asp and Lys residues and Met oxidation were included as variable modification while carbamidomethylation, as fixed modification on Cys residues, were included when samples were treated with DTT and iodoacetamide.
3.2 Identification and characterization of HSA dimeric form

3.2.1 Identification of HSA dimeric form in plasma samples by Western Blot analysis

In order to evaluate the HSA dimerization occurs in patients with cirrhosis, plasma samples from three cirrhotic patients and three healthy subjects were subjected to native protein electrophoresis and western blot analyses. Sample were diluted 1:2 in double distilled water (DDW) and filtered through 0.22-µm filters (Millex GP). Total protein content of the sample was assessed through the Lowry method and the protein concentration was normalized; 168 µg of total plasma proteins of every sample were mixed with Laemmlı Buffer and loaded on a native 8% polyacrylamide gel. After separation proteins were blotted on a PVDF membrane (Millipore) and incubated overnight with rabbit anti-human albumin (1:8000; Dako). After incubation with horseradish peroxidase-conjugated anti-rabbit antibody, the immune complexes were detected by enhanced chemiluminescence with an ECL kit (Westar C, Cyanagen). The signal was acquired by exposing membranes on a ChemiDoc XRS System (Biorad).

3.2.2 Identification of HSA dimers by MALDI-ToF analysis

Serum sample was diluted (1:10) with water and ultrafiltrated at 4°C 4000 rpm for 1h [Amicon Ultra with 3 K cut-off (Millipore)], after that a washing step was performed. Once concentrated and purified sample was subjected to MALDI-ToF MS using a Voyager DE Pro (Applied Biosystems, Foster City, CA) equipped with a pulsed N2 laser operating at 337 nm. Positive ion spectra were acquired in linear mode over an \( m/z \) range from 50,000-200,000 using a 25,000 V accelerating voltage, a 22,000-V grid voltage, and a delay extraction time of 150 ns. The spectrum for each spot was obtained by averaging the result of 120 laser shots. External mass calibration was performed using the single- and double-charged ions of bovine serum albumin (Sigma–Aldrich). The analysis was performed by spotting on the target plate 1 µL of the sample mixed with an equal volume of the matrix solution, 30 mg/mL sinapinic acid in 1:1 (v/v) ACN/H₂O containing 0.1% (v/v) trifluoroacetic acid (TFA).

3.2.3 Assessment of the site involved in the dimerization process.

A serum sample containing approximately 95 µg of HSA was diluted 1:10 with 10 mM ammonium bicarbonate, pH 8.0 and digested using trypsin at a mass ratio of 1:50 enzyme/protein overnight at 37 °C. The hydrolysis was stopped by addition of 1%
formic acid (FA) and the sample was analyzed by a 2D-LC-ESI/MSMS approach described in section 3.1.3.

3.2.4 Role of oxidative stress in the dimer formation
Serum sample from an healthy volunteer was incubated at 37°C in the presence of \( t \)-butyl hydro peroxide (\( t \)-BuOOH).

Time dependent experiments were performed incubating serum (99 µL) with \( t \)-BuOOH (1 µL) to reach the oxidative agent final concentration of 0.2 and 2 mM. The oxidation process was monitored at 0.5, 1, 2, 3, 6, 24 and 48 h. For the dose dependent studies serum (99 µL) was incubated for 24 h with \( t \)-BuOOH (1 µL) to reach the oxidative agent final concentration 0, 0.05, 0.075, 0.1, 0.2, 1 and 5 mM. The incubation mixture was diluted (1:100) with water at fixed times and 15 µL were analyzed with the LC-ESI-MS approach.

3.2.5 HSA Dimeric form analysis by LC-ESI-MS
Serum sample preparation was performed as described in section 3.1.1 HSA dimeric form LC-ESI-MS analysis was performed as described in section 3.1.2. The dimeric HSA detection was obtained deconvoluting the baseline-subtracted spectrum (\( m/z \) 1084–1534) acquired on HSA peak apex in the mass range 61,500–138,000 Da.

The isoforms relative percentage abundance was calculated by dividing the isoform intensity obtained from the deconvoluted and baseline-subtracted spectrum by the sum of all isoform intensities and multiplying them by 100. Data were analyzed by Excel.

For the oxidative experiments, described in section 3.2.4, the relative abundance of each dimeric isoform was expressed in percentage and calculated following this formula:

\[
\frac{HSA\ dimer\ area}{HSA\ dimer\ area + HSA\ monomer\ area} \times 100
\]

The HSA dimer area consists in the sum of all dimeric HSA isoforms while the HSA monomer area intends the sum of all the monomeric HSA isoforms.
4. Evaluation of HSA functional alterations

Alterations of the metal binding activity of the N-terminal portion of HSA were evaluated through the measurement of the circulating level of Ischemia modified Albumin (IMA).

4.1 Evaluation of IMA and IMAR in human plasma samples

IMA levels were measured through the Albumin Cobalt binding test (ACB) in heparinized plasma samples. This colorimetric assay allow to quantify the ability of HSA to chelate cobalt. Briefly, 200µl of plasma samples were incubated with 0.1% cobalt chloride for 10 minutes. Afterward dithiothreitol (1.5 g/L, 2 min) was added and the mixture diluted in saline solution prior to measurement at 470 nm in a spectrophotometer (BioMate3; Thermo Spectronic). The IMA level in each samples was expressed as relative absorbance unit (ABSU), while the IMA to albumin ratio (IMAr) was calculated by dividing the IMA value for total serum albumin concentration as measured by standard commercial kit.

4.2 Experimental animal studies

The relationship between bacterial infections and plasma IMA levels was also evaluated in experimental cirrhosis. Briefly, six male Wistar rats (Charles River Laboratories), weighing 175–200 g, were housed in a temperature (22–24°C) and moisture (40–70%) controlled room with a 12-h light-dark cycle. Cirrhosis induction was carried out through CCl₄ inhalation following a validated protocol, at the same time phenobarbital (0.3 g/L in drinking water) was administered to shorten the time required to induce cirrhosis. After 14–18 weeks of CCl₄ administration, all rats developed ascites as confirmed by ultrasound examination. Cirrhotic rats and six age and sex matched healthy rats received a single intravenous injection through the tail vein of lipopolysaccharide (10 mg/kg LPS, Escherichia coli 0127-B8; Sigma Co). After 2 and 6h from LPS administration, 0.5 ml of blood was withdrawn from the tail vein and plasma IMA was evaluated as above described. All animal experiments were conducted according to the guidelines for the care and use of laboratory animals and were approved by the ethical committee of our Institution.
5. Statistical analysis

All continuous variables were expressed as mean and standard deviation, while categorical data were reported as frequencies [n(%)]. Comparisons between categorical variables were made by means of the chi-square test while differences in HSA isoform relative abundance between control subjects, outpatients, and hospitalized patients with cirrhosis were assessed by one-way analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons. The comparison of IMA and IMAr levels in healthy controls and cirrhotic patients was made by means of Student’s t-test. The relationship between relative abundance of HSA isoforms, IMA, IMAr and MELD and Child-Pugh scores was evaluated by the Spearman’s rho correlation analysis.

The association between HSA isoforms relative amount, IMA, IMAr, age, MELD and Child-Pugh scores, and specific clinical complication (ascites, renal impairment, and bacterial infection) in hospitalized patients was assessed by means of Student’s t-test. Multivariate analysis were performed with logistic regression with Wald’s backward method, where the dependent variable was the presence of a specific complication. Only variables significantly associated to the specific outcome (p<0.005) at univariate analysis were entered in the multivariate analysis.

Survival analysis was performed by means of univariate Cox regression model. For each parameters found to be significantly associated to 1-year survival the best cut-off was calculated through the Receiver Operating Characteristics (ROC) curve analysis. Then survival curves were plotted according the Kaplan Meier method, while the survival rates of different groups were compared by means of Log Rank test.

All tests were two sided, and values of p less than 0.05 were considered statistically significant. All analyses were performed using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp., Armonk, NY).
RESULTS

1 Patients

One hundred thirty-three cirrhotic patients (59 ± 11 years; 64% males) admitted to the hepatological unit of the S. Orsola-Malpighi University Hospital for the onset of complications of cirrhosis were consecutively enrolled. Thirty-five patients with stable cirrhosis attending to the outpatients clinic (56 ± 13, 71% males) and forty-four healthy volunteers (57 ± 9 years; 58% males) were also recruited. Data regarding etiology of cirrhosis, causes of hospitalization, biochemical parameters, MELD and Child-Pugh scores of patients were reported in Table 2. The more frequent complications registered at admission were ascites (66 pts), bacterial infection (41 pts) and renal impairment with creatinine>1.5 mg/dL (33pts). Forty-seven (35%) patients with decompensated cirrhosis and 9 (36%) outpatients had type II diabetes mellitus while 14 (10%) patients with decompensated cirrhosis and 6 (21%) outpatients had HCC meeting the Milan criteria.
Table 2. *Clinical characteristics of hospitalized patients and outpatients with cirrhosis enrolled in the study.*

<table>
<thead>
<tr>
<th></th>
<th>Hospitalized patients N=133</th>
<th>Outpatients N=35</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Etiology of cirrhosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral</td>
<td>74 (55%)</td>
<td>19 (54%)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>29 (22%)</td>
<td>7 (20%)</td>
</tr>
<tr>
<td>Other</td>
<td>30 (23%)</td>
<td>9 (26%)</td>
</tr>
<tr>
<td><strong>Complication at hospital admission</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td>66 (50%)</td>
<td>-</td>
</tr>
<tr>
<td>Bacterial infection</td>
<td>41 (31%)</td>
<td>-</td>
</tr>
<tr>
<td>Renal impairment¹</td>
<td>33 (25%)</td>
<td>-</td>
</tr>
<tr>
<td>Encephalopathy grade III-IV</td>
<td>13 (10%)</td>
<td>-</td>
</tr>
<tr>
<td>Variceal Bleeding</td>
<td>9 (7%)</td>
<td>-</td>
</tr>
<tr>
<td>HCC meeting the Milan criteria</td>
<td>14 (10%)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum bilirubin (mg/dL)</td>
<td>4.4±5.8</td>
<td>2.1±3.3</td>
</tr>
<tr>
<td>Serum albumin (g/dL)</td>
<td>3.3±0.7</td>
<td>3.5±0.6</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.2±0.7</td>
<td>1.2±0.9</td>
</tr>
<tr>
<td>Serum sodium (mmol/L)</td>
<td>137±4</td>
<td>138±4</td>
</tr>
<tr>
<td>INR²</td>
<td>1.56±0.45</td>
<td>1.25±0.19</td>
</tr>
<tr>
<td><strong>Prognostic scores</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child-Pugh class (A/B/C)</td>
<td>27/77/29 (20/58/22)</td>
<td>19/12/4 (54/34/12)</td>
</tr>
<tr>
<td>Child-Pugh score</td>
<td>8±2</td>
<td>7±2</td>
</tr>
<tr>
<td>MELD score</td>
<td>17±7</td>
<td>12±5</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>47 (35%)</td>
<td>9 (26%)</td>
</tr>
<tr>
<td>HCC meeting the Milan criteria</td>
<td>14 (10%)</td>
<td>6 (21%)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation or frequencies (%). ¹Renal impairment = serum creatinine > 1.5 mg/dL ²Prothrombin time international normalized ratio.
2 Structural alterations to the HSA molecule

2.1 Monomeric HSA isoforms in healthy subjects and hospitalized patients with cirrhosis

2.1.1 Monomeric HSA isoforms in healthy subjects

According to the results previously provided by our group in a smaller population\(^7\), beyond the native and unchanged HSA form, other 7 monomeric isoforms with one or two alterations were identified: HSA-DA, HSA-L, HSA+CYS, HSA+SO\(_2\)H, HSA+GLYC, HSA+CYS-DA, HSA+CYS+GLYC (Figure 8a). The molecular weight of each HSA isoform and the corresponding functional alteration is reported in Table 3. The hypothesized sites and types of structural alterations previously reported were further confirmed by protein digestion followed by a 2D LC-ESI-MSMS analysis.

Table 3. Human serum albumin (HSA) isoforms identified in plasma samples: molecular weight, structural alterations and functional consequences.

<table>
<thead>
<tr>
<th>MW (Da)</th>
<th>Isoforms</th>
<th>Structural alteration</th>
<th>Functional consequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-180</td>
<td>HSA-DA</td>
<td>N-terminal truncated (-Asp-Ala)</td>
<td>Loss of chelating function, loss of free radical scavenging activity</td>
<td>71</td>
</tr>
<tr>
<td>-110</td>
<td>HSA-L</td>
<td>C-terminal truncated (-Leu)</td>
<td>Impaired protein stability, shorter half-life</td>
<td>30</td>
</tr>
<tr>
<td>-60</td>
<td>HSA+CYS-DA</td>
<td>Cysteinylated and N-terminal truncated (-Asp-Ala)</td>
<td>Loss of antioxidant activity, loss of chelating function</td>
<td>34, 71</td>
</tr>
<tr>
<td>66439</td>
<td>Native HSA</td>
<td>Native form of HSA with fully preserved non-oncotic activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+30</td>
<td>HSA+SO(_2)H</td>
<td>Sulfinylated</td>
<td>Irreversible oxidation of Cys-34 residue</td>
<td>34, 30</td>
</tr>
<tr>
<td>+120</td>
<td>HSA+CYS</td>
<td>Cysteinylated</td>
<td>Loss of antioxidant activity</td>
<td>34, 74</td>
</tr>
<tr>
<td>+160</td>
<td>HSA+GLY</td>
<td>Glycosylated</td>
<td>Impaired binding activity, conformational alteration</td>
<td>66</td>
</tr>
<tr>
<td>+280</td>
<td>HSA+CYS+GLY</td>
<td>Cysteinylated and glycosylated</td>
<td>Impaired antioxidant and binding activity, conformational alteration</td>
<td>34, 74, 66</td>
</tr>
</tbody>
</table>
Figure 8. Deconvoluted ESI-MS spectra from an healthy control (A) and a cirrhotic patient (B). In each mass spectra, besides the native HSA, additional seven isoforms were detected: HSA-DA, HSA-L, HSA+CYS, HSA+SO$_2$H, HSA+GLYC, HSA+CYS-DA, HSA+CYS+GLYC.
2.1.2 Monomeric HSA isoforms in patients with cirrhosis

The HSA isoforms identified in the control group were detected in cirrhotic patients as well (Figure 8B). Otherwise, the evaluation of the relative amount of each identified isoform showed that impaired HSA isoforms were more abundant in hospitalized patients and outpatients with cirrhosis than in healthy controls. Among these isoforms the cysteinylated (HSA+CYS, HSA+CYS+GLYC, HSA+CYS-DA) and glycosylated (HSA+GLYC) forms were the most abundant (Table 4). As a result, a significant reduction of the native, unchanged HSA relative abundance was detected in both groups of patients with respect to control population (Figure 9).

![Graph](image)

**Figure 9.** Relative amount of native HSA isoform in healthy controls, outpatients, and hospitalized patients with cirrhosis expressed as individual values and box plots of mean, range, and 95% confidence interval.

Although almost all HSA isoforms were more abundant in hospitalized patients than outpatients only the HSA+GLYC+CYS isoform reached the statistical significance (Table 4).
Table 4. Relative abundance of HSA isoforms in healthy controls (n=44), outpatients (n=35) and hospitalized patients with cirrhosis (n=133).

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Healthy Controls N=44</th>
<th>Outpatients N=35</th>
<th>Hospitalized Patients N=133</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-DA (%)</td>
<td>2.5±0.7</td>
<td>2.4±0.7</td>
<td>2.3±0.9</td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>3.1±1.2</td>
<td>2.4±1.3</td>
<td>2.3±1.9</td>
</tr>
<tr>
<td>HSA+CYS-DA (%)</td>
<td>1.9±0.3</td>
<td>2.3±0.4*</td>
<td>2.1±0.8*</td>
</tr>
<tr>
<td>Native HSA</td>
<td>52.4±2.6</td>
<td>45.9±5.6*</td>
<td>43.2±6.2*</td>
</tr>
<tr>
<td>HSA+SO$_2$H (%)</td>
<td>7.3±1.3</td>
<td>8.0±1.1</td>
<td>7.9±2.4</td>
</tr>
<tr>
<td>HSA+CYS (%)</td>
<td>19.9±4.4</td>
<td>24.3±7.3*</td>
<td>26.0±6.3*</td>
</tr>
<tr>
<td>HSA+GLYC (%)</td>
<td>9.1±1.3</td>
<td>9.6±2.6</td>
<td>9.8±3.1*</td>
</tr>
<tr>
<td>HSA+CYS+GLYC (%)</td>
<td>3.8±0.8</td>
<td>5.1±1.6*</td>
<td>6.3±2.1*</td>
</tr>
</tbody>
</table>

HSA-DA: N-terminal truncated (-Asp-Ala); HSA-L: C-terminal truncated (-Leu); HSA+CYS-DA: cysteinylated and N-terminal truncated (-Asp-Ala); native HSA: native form of HSA; HSA+SO$_2$H: sulfinilated; HSA+CYS: cysteinylated; HSA+GLYC: glycosylated; HSA+CYS+GLYC: cysteinylated and glycosylated. Data were expressed as mean ± SD. Each isoform relative abundance was calculated as percent over total HSA isoforms. *P<0.05 versus healthy controls. $^1$P<0.05 versus outpatients with cirrhosis.

2.1.3 Correlation between HSA isoforms and disease severity

Following the correlation analysis no association were found between the HSA-DA, HSA-L, and both MELD and Child-Pugh scores. On the contrary, all the HSA isoforms with structural changes at the Cys-34 residue level (HSA+CYS, HSA+CYS-DA, HSA+CYS-GLYC, and HSA+SO$_2$H) directly correlated with the MELD score (Table 5). The same association was found with the Child-Pugh score, with the exception of the HSA+CYS and HSA+CYS+GLYC isoforms. Contrariwise, an inverse correlation was found between the HSA+GLYC isoform and MELD or Child-Pugh scores. As a
result of the above reported association, a strong negative correlation was found between the relative abundance of the native HSA isoform and both prognostic scores.

**Table 5. Analysis of the correlation between relative abundance of HSA isoforms and MELD and Child Pugh Scores in the entire population of cirrhotic patients (n=168)**

<table>
<thead>
<tr>
<th>HSA isoforms</th>
<th>MELD Correlation coefficient</th>
<th>MELD P value</th>
<th>Child-Pugh Correlation coefficient</th>
<th>Child-Pugh P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-DA (%)</td>
<td>0.045</td>
<td>0.584</td>
<td>0.003</td>
<td>0.970</td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>-0.040</td>
<td>0.627</td>
<td>-0.057</td>
<td>0.478</td>
</tr>
<tr>
<td>HSA+CYS-DA (%)</td>
<td>0.266</td>
<td><strong>0.001</strong></td>
<td>0.201</td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>-0.318</td>
<td>&lt;0.001</td>
<td>-0.263</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>HSA+SO₂H (%)</td>
<td>0.259</td>
<td><strong>0.001</strong></td>
<td>0.265</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>HSA+CYS</td>
<td>0.219</td>
<td><strong>0.007</strong></td>
<td>0.149</td>
<td>0.064</td>
</tr>
<tr>
<td>HSA+GLYC</td>
<td>-0.220</td>
<td><strong>0.007</strong></td>
<td>-0.195</td>
<td><strong>0.015</strong></td>
</tr>
<tr>
<td>HSA+CYS+GLYC</td>
<td>0.181</td>
<td><strong>0.026</strong></td>
<td>0.120</td>
<td>0.136</td>
</tr>
</tbody>
</table>

_HSA-DA_: N-terminal truncated (-Asp-Ala); _HSA-L_: C-terminal truncated (-Leu); _HSA+CYS-DA_: cysteinylated and N-terminal truncated (-Asp-Ala); _native HSA_: native form of HSA; _HSA+SO₂H_: sulfinitilated; _HSA+CYS_: cysteinylated; _HSA+GLYC_: glycosylated; _HSA+CYS+GLYC_: cysteinylated and glycosylated

2.1.4 Relationship between HSA isoforms and complications of liver cirrhosis

We first compared the relative abundance of the monomeric and dimeric HSA isoforms in patients with or without the following complications: ascites, renal impairment and bacterial infection (Table 6). As only few patients presented variceal bleeding, HCC and hepatic encephalopathy (Table 2), we decided to exclude these complications from the
analysis. For each complication, following univariate analysis a multivariate logistic regression was performed in order to identify HSA isoforms independently associated to the specific complication (Table7).

**Ascites.** As expected, patients with ascites showed higher value of MELD and Child-Pugh scores, furthermore they presented a significantly higher relative abundance of HSA-DA, HSA+CYS-DA, HSA+SO₂H and a significant reduced abundance of HSA+GLYC respect to those without the complication. Also the native, unchanged, HSA isoform was significantly lower in patients with ascites with respect to patients not presenting this complication at study inclusion. All variable significantly associated to the presence of ascites at univariate analysis were entered in the multivariate logistic regression model with the exception of the Child-Pugh score which account for the presence of ascites in its calculation. The analysis showed that the relative amount of the HSA+CYS-DA isoform is independently associated to presence of ascites.

**Renal Impairment.** Patients with serum creatinine level higher than 1.5 mg/dL have an higher MELD and Child Pugh scores, instead for the isoforms. Additionally they presented higher relative amount of the HSA+CYS, HSA+CYS-DA, HSA+SO₂H and HSA+GLYC isoforms with respect to patients without renal impairment. As for patients with ascites, the native HSA isoform was significantly reduced in patients with renal impairment. The multivariate logistic regression analysis, in which the MELD score, which account for serum creatinine level was not considered, showed that the HSA+CYS and HSA+SO₂H isoforms were independently associated with the presence of renal impairment.

**Bacterial Infection.** Although not statistically significant, prognostic scores tended to be higher in patients with bacterial infection with respect to patients without bacterial infection. On the contrary the relative abundance of HSA-L and HSA+CYS-DA isoforms were significantly increased in patients with bacterial infections, while the native HSA resulted significantly reduced. From the multivariate logistic regression analysis, only the HSA+CYS-DA isoform resulted to be independently associated with the presence of bacterial infections.
Table 6. Univariate analysis of the association between age, disease severity scores and human serum albumin (HSA) isoforms relative abundance with ascites, renal dysfunction, or bacterial infection in hospitalized cirrhotic patients.

<table>
<thead>
<tr>
<th></th>
<th>Ascites Yes (N = 66)</th>
<th>Ascites No (N = 67)</th>
<th>P</th>
<th>Renal impairment Yes (N = 33)</th>
<th>Renal impairment No (N = 100)</th>
<th>P</th>
<th>Bacterial infection Yes (N = 41)</th>
<th>Bacterial infection No (N = 92)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58 ± 11</td>
<td>61 ± 12</td>
<td>0.092</td>
<td>59 ± 10</td>
<td>60 ± 12</td>
<td>0.733</td>
<td>61 ± 13</td>
<td>59 ± 11</td>
<td>0.255</td>
</tr>
<tr>
<td>MELD score</td>
<td>18.6 ± 6.9</td>
<td>15.1 ± 7.2</td>
<td>0.001</td>
<td>22.5 ± 7.1</td>
<td>15.02 ± 6.3</td>
<td>0.014</td>
<td>18.6 ± 7.1</td>
<td>16.1 ± 7.2</td>
<td>0.286</td>
</tr>
<tr>
<td>Child-Pugh score</td>
<td>9.1 ± 1.5</td>
<td>7.4 ± 1.8</td>
<td>0.006</td>
<td>8.9 ± 2.0</td>
<td>8.1 ± 1.8</td>
<td>0.001</td>
<td>8.5 ± 1.8</td>
<td>8.1 ± 1.9</td>
<td>0.073</td>
</tr>
<tr>
<td>HSA-DA (%)</td>
<td>2.6 ± 0.9</td>
<td>2.1 ± 0.9</td>
<td>0.001</td>
<td>2.4 ± 0.9</td>
<td>2.3 ± 0.9</td>
<td>0.413</td>
<td>2.4 ± 1.0</td>
<td>2.3 ± 0.9</td>
<td>0.354</td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>2.3 ± 2.0</td>
<td>2.4 ± 1.9</td>
<td>0.936</td>
<td>2.0 ± 1.0</td>
<td>2.5 ± 2.1</td>
<td>0.203</td>
<td>1.9 ± 1.0</td>
<td>2.5 ± 2.2</td>
<td>0.040</td>
</tr>
<tr>
<td>HSA+CYS–DA (%)</td>
<td>2.5 ± 0.9</td>
<td>1.8 ± 0.7</td>
<td>0.001</td>
<td>2.6 ± 0.9</td>
<td>2.0 ± 0.7</td>
<td>0.001</td>
<td>2.4 ± 1.0</td>
<td>2.0 ± 0.7</td>
<td>0.006</td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>42.1 ± 6.4</td>
<td>44.2 ± 5.6</td>
<td>0.049</td>
<td>39.9 ± 5.9</td>
<td>44.3 ± 5.9</td>
<td>0.001</td>
<td>41.2 ± 6.3</td>
<td>44.1 ± 5.9</td>
<td>0.017</td>
</tr>
<tr>
<td>HSA+SO₂H (%)</td>
<td>8.6 ± 2.1</td>
<td>7.1 ± 2.4</td>
<td>0.001</td>
<td>8.7 ± 2.4</td>
<td>7.6 ± 2.3</td>
<td>0.021</td>
<td>8.5 ± 2.6</td>
<td>7.6 ± 2.2</td>
<td>0.067</td>
</tr>
<tr>
<td>HSA+CYS (%)</td>
<td>26.4 ± 6.6</td>
<td>25.5 ± 6.0</td>
<td>0.435</td>
<td>29.0 ± 7.2</td>
<td>25.0 ± 5.7</td>
<td>0.008</td>
<td>27.5 ± 6.4</td>
<td>25.3 ± 6.2</td>
<td>0.074</td>
</tr>
<tr>
<td>HSA+GLYC (%)</td>
<td>9.2 ± 2.6</td>
<td>10.5 ± 3.5</td>
<td>0.019</td>
<td>8.9 ± 2.8</td>
<td>10.2 ± 3.2</td>
<td>0.041</td>
<td>9.2 ± 2.9</td>
<td>10.1 ± 3.2</td>
<td>0.108</td>
</tr>
<tr>
<td>HSA+CYS+GLYC (%)</td>
<td>6.1 ± 2.0</td>
<td>6.4 ± 2.1</td>
<td>0.518</td>
<td>6.5 ± 1.7</td>
<td>6.2 ± 2.2</td>
<td>0.468</td>
<td>6.8 ± 2.1</td>
<td>6.0 ± 2.0</td>
<td>0.070</td>
</tr>
</tbody>
</table>

MELD: model for end stage liver disease; HSA-DA: N-terminal truncated (-Asp-Ala); HSA-L: C-terminal truncated (-Leu); HSA+CYS-DA: cysteinylated and N-terminal truncated (-Asp-Ala); native HSA: native form of HSA; HSA+SO₂H: sulfinylated; HSA+CYS: cysteinylated; HSA+GLYC: glycosylated; HSA+CYS+GLYC: cysteinylated and glycosylated.
Table 7. Binary logistic regression model with backward selection of variables independently associated to ascites, renal impairment and bacterial infection in hospitalized cirrhotic patients.

<table>
<thead>
<tr>
<th></th>
<th>Ascites(^a)</th>
<th>Renal impairment(^b)</th>
<th>Bacterial infection(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OR (95% CI)</strong></td>
<td><strong>p</strong></td>
<td><strong>OR (95% CI)</strong></td>
<td><strong>p</strong></td>
</tr>
<tr>
<td>HSA+CYS–DA</td>
<td>2.36 (1.25-4.46)</td>
<td>0.008</td>
<td>-</td>
</tr>
<tr>
<td>HSA+SO(_2)H</td>
<td>-</td>
<td>-</td>
<td>1.36 (1.11-1.66)</td>
</tr>
<tr>
<td>HSA+CYS</td>
<td>-</td>
<td>-</td>
<td>1.15 (1.06-1.24)</td>
</tr>
</tbody>
</table>

\(^a\)Variables entered on step one: MELD score, HSA-DA, HSA+CYS-DA, HSA, HSA+SO\(_2\)H, HSA+GLYC. \(^b\)Variables entered on step one: Child-Pugh score, HSA+CYS-DA, HSA, HSA+SO\(_2\)H, HSA+CYS, HSA+GLYC. \(^c\)Variables entered on step one: HSA-L, HSA+CYS-DA, HSA

2.1.5 Relationship between HSA isoforms and diabetes mellitus

A comparison between relative abundances of HSA isoforms was performed among hospitalized cirrhotic patients with and without type 2 diabetes mellitus and control population. A reduction of the native, unchanged HSA was observed in cirrhotic patients with diabetes with respect to those without, the same trend was observed for the truncated isoforms as HSA-DA and HSA-L in patients with diabetes with respect to those without. As expected, the glycosylated isoforms (HSA+GLYC and HSA+CYS+GLYC) showed an higher relative abundance in diabetic patients with respect to patients with preserved glycemic control, instead no differences were observed for the isoforms with oxidation of Cys-34 residue among the two group of hospitalized patients (Table 8).
Table 8. Relative abundance of human serum albumin (HSA) isoforms in healthy controls and hospitalized cirrhotic patients divided according to the presence or not of type II diabetes mellitus.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 44)</th>
<th>Hospitalized cirrhotic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without diabetes (n = 86)</td>
</tr>
<tr>
<td>HSA–DA (%)</td>
<td>2.35 ± 0.45</td>
<td>2.51 ± 0.85</td>
</tr>
<tr>
<td>HSA–L (%)</td>
<td>2.94 ± 1.11</td>
<td>2.69 ± 2.27</td>
</tr>
<tr>
<td>HAS+CYS–DA (%)</td>
<td>1.67 ± 0.21</td>
<td>2.21 ± 0.85§</td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>50.70 ± 4.70</td>
<td>44.37 ± 6.08§</td>
</tr>
<tr>
<td>HSA+SO₂H (%)</td>
<td>6.90 ± 1.03</td>
<td>8.16 ± 2.34</td>
</tr>
<tr>
<td>HSA+CYS (%)</td>
<td>22.25 ± 4.84</td>
<td>25.28 ± 6.32</td>
</tr>
<tr>
<td>HSA+GLYC (%)</td>
<td>8.99 ± 1.28</td>
<td>9.21 ± 2.78</td>
</tr>
<tr>
<td>HSA+CYS+GLYC (%)</td>
<td>4.17 ± 1.34</td>
<td>5.43 ± 1.37§</td>
</tr>
</tbody>
</table>

HSA–DA: N-terminal truncated (-Asp-Ala); HSA–L: C-terminal truncated (-Lys); HSA+CYS–DA: cysteinylated and N-terminal truncated (-Asp-Ala); native HSA: native form of HSA; HSA+SO₂H: sulfinylated; HSA+CYS: cysteinylated; HSA+GLYC: glycosylated; HSA+CYS+GLYC: cysteinylated and glycosylated. *p<0.020 vs cirrhotics without diabetes; §p<0.050 vs controls.

2.1.6 Monomeric HSA isoforms and 1-year survival

After 1-year of follow-up 77 (60%) of hospitalized patients were still alive. In a preliminary analysis the Cox proportional hazard method was used to compare the association between HSA isoforms or serum albumin concentration and survival rate. Among all the HSA isoforms, the HSA+CYS–DA and the native HSA significantly re stratified the risk of death within 1 year (Table 9).
Table 9. Univariate Cox regression analysis of the association between HSA isoforms, serum albumin concentration and 1-years survival in admitted patients.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin (g/dl)</td>
<td>0.877</td>
<td>0.575-1.337</td>
<td>0.541</td>
</tr>
<tr>
<td>HSA-DA (%)</td>
<td>1.293</td>
<td>0.962-1.737</td>
<td>0.088</td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>0.931</td>
<td>0.777-1.116</td>
<td>0.439</td>
</tr>
<tr>
<td>HSA+CYS-DA (%)</td>
<td>1.566</td>
<td>1.113-2.203</td>
<td>0.010</td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>0.944</td>
<td>0.900-0.990</td>
<td>0.019</td>
</tr>
<tr>
<td>HSA+SO$_2$H (%)</td>
<td>1.106</td>
<td>0.986-1.241</td>
<td>0.085</td>
</tr>
<tr>
<td>HSA+CYS (%)</td>
<td>1.021</td>
<td>0.974-1.069</td>
<td>0.389</td>
</tr>
<tr>
<td>HSA+GLYC (%)</td>
<td>1.001</td>
<td>0.917-1.092</td>
<td>0.986</td>
</tr>
<tr>
<td>HSA+CYS+GLYC (%)</td>
<td>1.095</td>
<td>0.964-1.244</td>
<td>0.163</td>
</tr>
</tbody>
</table>

HSA–DA: N-terminal truncated (-Asp-Ala); HSA-L: C-terminal truncated (-Lys); HSA+CYS-DA: cysteinylated and N-terminal truncated (-Asp-Ala); native HSA: native form of HSA; HSA+SO$_2$H: sulfinylated; HSA+CYS: cysteinylated; HSA+GLYC: glycosylated; HSA+CYS+GLYC: cysteinylated and glycosylated.

Then, we categorized HSA+CYS-DA and the native HSA in relation to their best-cutoff calculated by the ROC curve analysis. Finally survival curves for HSA+CYS-DA, native HSA and serum albumin concentration were plotted according to the Kaplan Meyer’s method followed by the log-rank test to compare the survival rate. Cirrhotic patients presenting a relative amount of HSA+CYS-DA below 2.18% displayed a significant higher survival (10.4 ± 0.37 vs. 7.72 ± 0.64 months; P=0.001) with respect to patients with higher value (Figure 10A). Also patients with a relative amount of native HSA higher than 44.38% presented a longer survival with respect to those with a lower amount (10.30 ± 0.48 vs 8.55 ± 0.50 months; P=0.002) (Figure 10B). The best cut-off of total serum albumin concentration for 1-year survival was 4.05 g/dl in our series. The
Kaplan Meier survival analyses showed that cirrhotic patients with total albumin concentration below or equal 4.05 g/dl had a worse survival with respect to patients with values above the cut-off, however this result did not achieve a statistical significance (10.16 ± 0.65 vs 9.06 ± 0.43 months; P=0.063) (Figure 10C).

**Figure 10.** Kaplan-Meier’s survival curves for HSA+CYS-DA (A), native HSA (B), and serum albumin concentration (C) in hospitalized cirrhotic patients dichotomized according to their best cut-off evaluated by ROC curve analysis.
2.2 Dimeric HSA isoforms in healthy subjects and hospitalized patients with cirrhosis
The formation of HSA dimer was proposed in the literature as a structural modification of the molecule promoted by a prooxidant environment. Therefore we analyzed plasma samples from healthy controls and hospitalized patients with cirrhosis in order to evaluate if such alteration occurs in physiological condition and if cirrhosis could be associated to an increase of HSA dimers formation.

2.2.1 Identification of HSA dimers
The presence of dimeric HSA isoform was evaluated in plasma samples from healthy volunteers and cirrhotic patients by means of native gel electrophoresis followed by western blot analysis. From the enrolled population were undergone to electrophoresis in a polyacrylamide native gel. As showed in figure 11 the monomeric (expected molecular weight 65 KDa) and the dimeric (expected molecular weight 130 KDa) were identified in plasma samples from both controls and cirrhotic subjects. Additionally the signal intensity corresponding to the dimeric form seems to be higher in patients than in controls.

![Electrophoresis of a native gel run under not-denaturing and not-reducing conditions. Lines 1-3: plasma samples from healthy patients; lines 4-6 plasma samples from cirrhotic patients.](image)

Figure 11. Electrophoresis of a native gel run under not-denaturing and not-reducing conditions. Lines 1-3: plasma samples from healthy patients; lines 4-6 plasma samples from cirrhotic patients.
The presence of HSA dimeric form was further confirmed by MALDI TOF analysis. Indeed, in the mass spectrum of control and patients a signal at the expected molecular weight was identified. Moreover, comparable to the results provided by the western blot analysis, the dimers signal intensity was found to be higher in cirrhotic patients than healthy controls (Figure 12).

**Figure 12.** MALDI TOF mass spectra referred resulted from the analysis of plasma samples from an healthy control (Panel A) and a cirrhotic patient (Panel B).

2.2.2 Characterization of the molecular site involved in the dimerization process

The Cys-34 residue represent the main antioxidant site of the HSA molecule, therefore, it may be the target of oxidative modification leading to HSA dimers formation.
Namely, we hypothesized that the dimerization process may involve the formation of a disulfide bridge between two HSA molecules at the Cys-34 level.

Such hypothesis was confirmed by the LC-ESI-MSMS analysis in which, following plasma tryptic digestion, performed under non reducing conditions, in order to preserve the disulfide bonds, a dipeptide, arising from the interaction of two Cys-34 residues of two different HSA molecule was found (Figure 13). Confirming this result the dipeptide identified in the previous experiment disappeared when the enzymatic digestion was performed under reducing conditions.

**Figure 13.** Sequence and LC-ESI-MSMS mass spectra of the dipeptide generated by trypsin cleavage of a plasma sample.
2.2.3 Role of oxidative stress in the formation of HSA dimers

In order to confirm that oxidative stress plays a major role in the dimerization process of the HSA molecule, a plasma sample from an health volunteer was incubated with increased concentration of tert-Butyl hydroperoxide (t-BuOOH) for different time intervals. The analysis of the reaction products, performed by LC-ESI-MS, showed that, following 24 hours of incubation with 0.2 and 2.0 mM of t-BuOOH the relative abundance of the highly oxidize HSA-SO$_2$H isoform increased proportionally to the concentration of the oxidant agent. Overall a rise of all the monomeric cysteinylated HSA isoforms were observed in parallel to a marked reduction of native HSA (Figure 14). Analogously, also the percentage of dimeric HSA isoform increased in a time and dose dependent manner following exposure of the plasma sample to a pro oxidant environment (Figure 14).

![Figure 14](image.png)

**Figure 14.** Relative abundance of HSA monomeric isoforms in plasma sample incubated for 24 hours with t-BuOOH at different concentration (Panel A). HSA dimer relative abundance in plasma sample after 24 hours incubation with increasing concentration of t-BuOOH (Panel B).
2.2.4. Relative abundance of dimeric HSA isoforms in healthy subjects and hospitalized patients with cirrhosis

The relative abundance of the dimeric HSA isoforms was evaluated in the whole population of 133 cirrhotic patients and compared to that measured in 44 healthy controls. We focused on the three more represented dimeric HSA isoform and the corresponding monomeric isoforms. Namely we quantified the relative abundance of the isoforms resulted from the homodimerization of HSA-DA (dimeric HSA-DA, dHSA-DA), HSA-L (dimeric HSA-L, dHSA-L) and native HSA (dimeric HSA, dHSA) monomeric isoforms (Table 9).

Table 9. More represented human serum albumin (HSA) dimeric isoforms included in the analysis: molecular weight, structural alteration and functional consequences.

<table>
<thead>
<tr>
<th>MW (Da)</th>
<th>Isoforms</th>
<th>Structural alterations</th>
<th>Functional consequences</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>+66090</td>
<td>dHSA-DA</td>
<td>Homodimeric form of the N-terminal truncated(-Asp-Ala)</td>
<td>Loss of chelating function, free radical scavenging activity and antioxidant activity</td>
<td>71,84</td>
</tr>
<tr>
<td>+66230</td>
<td>dHSA-L</td>
<td>Homodimeric form of the C-terminal truncated(-Leu)</td>
<td>Impaired protein stability, shorter half-life, loss of antioxidant activity</td>
<td>30,84</td>
</tr>
<tr>
<td>+66460</td>
<td>dHSA</td>
<td>Homodimeric form of the native human serum albumin</td>
<td>Loss of antioxidant activity</td>
<td>84</td>
</tr>
</tbody>
</table>

*dHSA-DA*: homodimeric form of the N-terminal truncated human serum albumin; *dHSA-L*: homodimeric form of the C-terminal truncated human serum albumin; *dHSA*: homodimeric form of the native human serum albumin.

The analysis of the mass spectra revealed that the extent of HSA dimerization was significantly greater in patients than controls, while among the corresponding
monomeric isoforms, only the native unchanged HSA was significantly reduced in cirrhotic subjects (Table 10).

**Table 10. Relative abundance of dimeric human serum albumin (HSA) isoforms in healthy controls (n=44) and patients with cirrhosis (n=133).**

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (N = 44)</th>
<th>Patients with cirrhosis (N = 133)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-DA (%)</td>
<td>4.0 ± 0.6</td>
<td>4.3 ± 2.3</td>
<td>0.534</td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>5.2 ± 1.8</td>
<td>4.4 ± 6.2</td>
<td>0.281</td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>86.3 ± 2.3</td>
<td>80.9 ± 7.7</td>
<td>0.002</td>
</tr>
<tr>
<td>dHSA-DA (%)</td>
<td>2.8 ± 0.9</td>
<td>4.8 ± 2.1</td>
<td>0.000</td>
</tr>
<tr>
<td>dHSA-L (%)</td>
<td>1.1 ± 0.6</td>
<td>2.2 ± 1.3</td>
<td>0.000</td>
</tr>
<tr>
<td>dHSA (%)</td>
<td>0.6 ± 0.4</td>
<td>3.1 ± 3.7</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*HSA-DA*: N-terminal truncated (-Asp-Ala); *HSA-L*: C-terminal truncated (-Leu); *native HSA*: native form of HSA; *dHSA-DA*: homodimeric form of the N-terminal truncated human serum albumin; *dHSA-L*: homodimeric form of the C-terminal truncated human serum albumin; *dHSA*: homodimeric form of the native human serum albumin.

2.2.5. Correlation between dimeric HSA isoforms and disease severity

While no correlations were found between the monomeric HSA-DA and HSA-L isoforms and disease severity, as assessed by the MELD and Child Pugh scores, a negative correlation was found between the relative abundance of the native, unchanged, HSA and both prognostic scores, thus confirming the previous findings and supporting the idea that the more severe is cirrhosis, the lower is the circulating HSA with a fully preserved molecular structure.

Contrary to the monomeric isoforms, all the dimeric HSA isoforms significantly correlated with the MELD and Child-Pugh scores (Table 11).
Table 11. Analysis of correlation between relative abundance of human serum albumin (HSA) isoforms and MELD and Child-Pugh scores.

<table>
<thead>
<tr>
<th></th>
<th>MELD</th>
<th></th>
<th></th>
<th>Child-Pugh</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman rho</td>
<td>P</td>
<td>Spearman rho</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>HSA-DA (%)</td>
<td>0,091</td>
<td>0,315</td>
<td>0,088</td>
<td>0,334</td>
<td></td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>-0,205</td>
<td>0,023</td>
<td>-0,186</td>
<td>0,040</td>
<td></td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>-0,412</td>
<td>0,000</td>
<td>-0,358</td>
<td>0,000</td>
<td></td>
</tr>
<tr>
<td>dHSA-DA (%)</td>
<td>0,380</td>
<td>0,000</td>
<td>0,344</td>
<td>0,000</td>
<td></td>
</tr>
<tr>
<td>dHSA-L (%)</td>
<td>0,369</td>
<td>0,000</td>
<td>0,362</td>
<td>0,000</td>
<td></td>
</tr>
<tr>
<td>dHSA (%)</td>
<td>0,504</td>
<td>0,000</td>
<td>0,455</td>
<td>0,000</td>
<td></td>
</tr>
</tbody>
</table>

HSA-DA: N-terminal truncated (-Asp-Ala); HSA-L: C-terminal truncated (-Leu); native HSA: native form of HSA; dHSA-DA: homodimeric form of the N-terminal truncated human serum albumin; dHSA-L: homodimeric form of the C-terminal truncated human serum albumin; dHSA: homodimeric form of the native human serum albumin.

2.2.6 Relationships between HSA isoforms and liver cirrhosis complications

As for the monomeric HSA isoform, we evaluated the association between the relative abundance of the dimeric HSA isoform and the presence of ascites, renal impairment and bacterial infection (Table 12), which were the more frequent complication recorded at study inclusion (Table 2). Following univariate analysis, a multivariate logistic regression was performed to identify HSA dimeric and/or monomeric isoforms independently associated to the presence of clinical complications of cirrhosis.
Ascites. Besides the higher prognostic scores, as reported before, patients with ascites had significantly greater relative amounts of dHSA-DA isoform than compensated patients, while the native HSA was significantly lower (Table 12). The multivariate logistic regression analysis, including as covariates the native HSA, the dHSA-DA isoform and MELD score showed that the dHSA-DA isoform was independently associated with the presence of ascites irrespective of the severity of cirrhosis (OR 1.321; CI 95% 1.071-1.630; p=0.009).

Renal impairment. Patients with renal impairment presented an higher relative abundance of the dHSA-DA and dHSA isoforms (Table 13), together with significantly increased MELD and Child-Pugh scores as previously reported. The multivariate logistic regression analysis including the native HSA, the dHSA-DA, the dHSA isoforms and Child-Pugh score showed that the dHSA-DA was independently associated with the presence of renal impairment, irrespective of the disease severity as assessed by the Child-Pugh score (OR 1.377; CI 95% 1.084-1.750; p=0.009).

Bacterial infection. Only the relative abundance of the dHSA-DA isoform was significantly increased in patients presenting bacterial infection at study inclusion. The subsequent multivariate analysis, including the MELD score as covariate, showed that such association was independent from the severity of the disease (OR 1.537; CI 95% 1.229-1.922; p<0.001).
Table 12. The relative abundances of human serum albumin (HSA) dimeric isoforms and their respective monomers in cirrhotic patients with or without ascites, renal dysfunction, or bacterial infection.

<table>
<thead>
<tr>
<th></th>
<th>Ascites</th>
<th>Renal impairment</th>
<th>Bacterial infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>(N = 66)</td>
<td>(N = 67)</td>
<td></td>
</tr>
<tr>
<td>HSA-DA (%)</td>
<td>4.7 ± 1.9</td>
<td>3.9 ± 2.6</td>
<td>0.050</td>
</tr>
<tr>
<td>HSA-L (%)</td>
<td>4.8 ± 8.2</td>
<td>4.1 ± 3.2</td>
<td>0.536</td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>79.0 ± 9.2</td>
<td>82.7 ± 5.3</td>
<td><strong>0.008</strong></td>
</tr>
<tr>
<td>dHSA-DA (%)</td>
<td>5.4 ± 2.0</td>
<td>4.2 ± 2.1</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>dHSA-L (%)</td>
<td>2.3 ± 1.1</td>
<td>2.1 ± 1.4</td>
<td>0.328</td>
</tr>
<tr>
<td>dHSA (%)</td>
<td>3.7 ± 4.8</td>
<td>2.6 ± 2.0</td>
<td><strong>0.138</strong></td>
</tr>
</tbody>
</table>

*HSA-DA*: N-terminal truncated (-Asp-Ala); *HSA-L*: C-terminal truncated (-Leu); native HSA: native form of HSA; *dHSA-DA*: homodimeric form of the N-terminal truncated human serum albumin; *dHSA-L*: homodimeric form of the C-terminal truncated human serum albumin; *dHSA*: homodimeric form of the native human serum albumin.
2.2.7 Association between dimeric HSA isoforms and 1-year patients survival

We compared the association between each dimeric HSA isoform with the survival rate by univariate analysis using the Cox proportional hazard method. Among the monomeric isoforms only the native HSA was significantly associated to 1-year survival, on the contrary the dimeric isoforms did not stratify the risk of death within 1-year, although the association between dHSA-DA and patients prognosis was very close to the statistical significance (Table 13).

Table 13. Univariate Cox regression analysis of the association between monomeric and dimeric HSA isoforms and 1-years survival in hospitalized patients with cirrhosis.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAS-DA (%)</td>
<td>1.085</td>
<td>0.994-1.085</td>
<td>0.068</td>
</tr>
<tr>
<td>HAS-L (%)</td>
<td>1.003</td>
<td>0.960-1.047</td>
<td>0.900</td>
</tr>
<tr>
<td>Native HSA (%)</td>
<td>0.974</td>
<td>0.949-0.999</td>
<td>0.040</td>
</tr>
<tr>
<td>dHSA-DA (%)</td>
<td>1.139</td>
<td>0.996-1.302</td>
<td>0.057</td>
</tr>
<tr>
<td>dHSA-L (%)</td>
<td>1.134</td>
<td>0.918-1.402</td>
<td>0.243</td>
</tr>
<tr>
<td>dHSA (%)</td>
<td>1.032</td>
<td>0.955-1.115</td>
<td>0.430</td>
</tr>
</tbody>
</table>

HAS-DA: N-terminal truncated (-Asp-Ala); HAS-L: C-terminal truncated (-Leu); native HSA: native form of HSA; dHSA-DA: homodimeric form of the N-terminal truncated human serum albumin; dHSA-L: homodimeric form of the C-terminal truncated human serum albumin; dHSA: homodimeric form of the native human serum albumin.

In order to plot survival curve the native HSA and the dHSA-DA isoforms were categorized according to their best cut-off determined through the ROC curve analysis. The survival analysis, performed according to the Kaplan Meier method, showed that patients with relative amount of dHSA-DA lower or equal than 4.74% had a significantly higher survival than their counterparts (10.22 ± 0.44 vs 8.28 ± 0.56
months, p=0.001) (Figure 15, panel A). Similarly, The analysis of survival curves confirmed the prognostic relevance of the native HSA isoform emerged from the Cox analysis. Namely, patient with relative amount of native HSA higher than 80.28% had a significant higher survival rate than patients with native HSA relative amount below or equal to 80.28% (9.94 ± 0.42 vs 8.33 ± 0.64 months, p=0.045) (Figure 15, panel B), thus confirming its prognostic role emerged from the analysis of all monomeric HSA isoforms previously performed.

Figure 15. Kaplan-Meier survival curves for dHSA-DA and native HSA dichotomized according to their best cut-off.
3. Functional alterations to the HSA molecule

3.1 Ischemia modified albumin (IMA) level in healthy controls and hospitalized patients with cirrhosis.

IMA levels were measured in 127 out of 133 hospitalized cirrhotic patients, for which heparinized plasma samples were available, and 44 healthy controls. Circulating IMA levels were significantly higher in cirrhotic patients as compared to healthy controls (0.511±0.118 vs 0.404±0.110 ABSU, \( P<0.001 \)) (Figure 16, panel A). After the normalization for the total serum albumin concentration, IMAr remained significantly higher in patients than controls (0.154±0.0058 vs 0.090±0.024 ABSU, \( P<0.001 \)) (Figure 16, panel B).

![Figure 16. Tukey boxes and whiskers plots (median and interquartile range), individual data of circulating IMA (a) expressed in arbitrary units (ABSU) and IMAr (IMA/serum albumin concentration) (b) levels in cirrhotic patients (n=127) and controls (n=44).](image)

3.2 Correlation between IMA and IMAr level and disease severity

Following the correlation analysis no significant associations were found between IMA levels and the severity of disease as assessed by both MELD and Child Pugh scores. Contrariwise a significant association was found between IMAr and the Child Pugh score, however this was an expected association since the calculation of both parameters accounts for the total serum albumin concentration (Table 13).
Table 13. Correlations between ischemia modified albumin (IMA), IMA to albumin ratio (IMAr) levels, MELD and Child-Pugh scores.

<table>
<thead>
<tr>
<th></th>
<th>MELD</th>
<th></th>
<th>Child-Pugh</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>p</td>
<td>Correlation coefficient</td>
<td>p</td>
</tr>
<tr>
<td>IMA (ABSU)</td>
<td>-0.016</td>
<td>0.856</td>
<td>0.123</td>
<td>0.169</td>
</tr>
<tr>
<td>IMAr (Ratio)</td>
<td>-0.046</td>
<td>0.606</td>
<td>0.204</td>
<td><strong>0.021</strong></td>
</tr>
</tbody>
</table>

3.3 Relationship between IMA, IMAr levels and complications of liver cirrhosis

IMA and IMAr levels were not associated to the presence of ascites or renal impairment (Table 14). On the contrary, IMA and IMAr were significantly associated to the presence of bacterial infections at hospital admission (Figure 17). Additionally, IMA and IMAr levels rise gradually from healthy controls to cirrhotic patients, reaching the highest value in cirrhotic patients with bacterial infection, being all comparisons statistically significant (Figure 17).

Table 14. Ischemia modified albumin (IMA) and IMA to serum albumin ratio (IMAr) levels in patients with or without ascites or renal impairment at hospital admission

<table>
<thead>
<tr>
<th></th>
<th>Ascites</th>
<th>Renal failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=62)</td>
<td>No (n=65)</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>IMA (ABSU)</td>
<td>0.51±0.12</td>
<td>0.507±0.115</td>
</tr>
<tr>
<td>IMAr (ratio)</td>
<td>0.16±0.06</td>
<td>0.151±0.060</td>
</tr>
</tbody>
</table>
Figure 17. Tukey boxes and whiskers plots (median and interquartile range), single values for each patient of IMA (panel A) and IMAR (panel B) of controls (n=44), cirrhotic patients (87), and cirrhotic patients with bacterial infections (40).

We then compared the discriminating performance against the presence of bacterial infection of all parameters we found to be significantly increased in infected patients, namely IMA, IMAr, CRP levels and CYS-DA relative abundance. The ROC curve analysis showed that IMA (AUROC 0.746, 95% CI 0.646-0.845) and CRP (AUROC 0.746, 95% CI 0.654-0.838) levels had a good diagnostic power, while IMAr (AUROC 0.722, 95% CI 0.627-0.818) and HAS+CYS-DA (AUROC 0.604, 95% CI 0.489-0.718) were found to be less efficient in discriminating the presence of bacterial infection.
Additionally the multivariate binary logistic regression showed that IMA (OR 2.411, 95% CI 1.520-3.824 p=0.001) and CRP (OR 1.203, 95% CI 1.013-1.427, P=0.035) were both independently associated to the presence of bacterial infection.

3.4 Association between IMA, IMAr and 1-year survival
The univariate analysis, performed through the Cox proportional hazard method, showed that IMA (OR 0.863, 95% CI 0.078-9.596, p=0.905) and IMAr (OR 0.784, 95% CI 0.007-92.155 p=0.920) levels were not associated to the overall 1-year survival rate. (Data are not shown).

3.5 IMA levels in rats with experimental cirrhosis and bacterial infection
In order to further confirm the association between the functional impairment of the N-terminal portion of the HSA molecule and the presence of bacterial infection, we measured IMA levels in cirrhotic rats in which bacterial infection were mimicked by the administration of a single sub-lethal dose of LPS. As shown in figure18, cirrhotic rats presented significantly higher baseline IMA levels with respect to healthy controls rats (0.484±0.097 vs 0.773±0.059 ABSU, p=0.001). After a single intravenous injection of LPS (10 mg/kg), while the IMA level at 2 and 6 hours only tended to increase in healthy rats without significant changes from baseline, it rose significantly up to 0.998 ± 0.087 ABSU (P = 0.001) in rats with cirrhosis.
Figure 18. Circulating Ischemia modified albumin (IMA) level in healthy (n=6) and cirrhotic rats (n=6) at baseline and after 2, and 6 hours from a single intravenous lipopolysaccharide (LPS, 10mg/kg) injection. Data were expressed as mean ± standard error mean.
DISCUSSION

In the present study the identification and quantification of structural and functional alterations to the HSA molecule were performed for the first time in a large cohort of patients with advanced cirrhosis admitted to the hospital for the onset of an acute clinical complication of the disease. By using advanced mass spectrometric technique, besides post-transcriptional changes to the albumin molecule previously reported in other clinical settings, new oxidative products, never reported before in patients with cirrhosis, were identified and characterized. Thus, the overall analysis of the albumin structural and functional integrity, performed in a large series of patients with cirrhosis, lead us the possibility to unravel the clinical and prognostic relevance of alteration in the HSA molecule which occurs during advanced chronic liver disease. Indeed, we found that specific pattern of molecular alterations, involving the Cys-34 residue, provided of antioxidant activity, but also other molecular sites, were associated to the severity of cirrhosis and to the presence of major clinical complication of the disease such as ascites, renal impairment and bacterial infection. More interesting, the residual amount of the native unchanged HSA, found to be reduced in the more severe patients, emerged as a potent predictor of 1-year mortality. Also the functional integrity of the N-terminal portion of the HSA molecule, provided with an indirect antioxidant activity, was found to be reduced in patients with cirrhosis. Contrary to what observed for the structural alteration, the N-terminal functional impairment of the HSA molecule was not influenced by the severity of cirrhosis nor by the presence of clinical complication of the disease such as ascites or renal impairment, while a strong association was found between a reduced chelating activity of HSA and the presence of bacterial infection.

The first important finding of the present study is that the molecular structure of HSA is extensively altered in patients with cirrhosis. Indeed, the HPLC-ESI-MS technique we employed, providing a spectrum of molecules with high resolution, discriminating between isoforms whose molecular weight differs by few Daltons, allowed an accurate characterization of the HSA circulating microheterogeneity in both patients and healthy subjects. Among the structural alterations revealed by the mass spectra, the cysteinylation was the more frequent. This alteration was found to occur alone or in combination with other molecular changes, among which the truncation of N-
terminal portion of the molecule and the glycosylation \textsuperscript{65-71}. This alteration likely results from the pro-oxidant environment, which develops in the plasma of patients with advanced cirrhosis. Indeed, in this setting circulating cysteine is oxidized to cysteine disulfide, which interacts with the free sulfhydryl group at the albumin Cys-34 site undergoing a disulfide exchange \textsuperscript{85}. The cysteinylation of the HSA molecule is therefore a result of this reversible oxidation. Since reduced Cys-34 accounts for the vast majority of the total plasma free sulfhydryl groups, a significant impairment of the circulating antioxidant system can be expected in the case of extensive oxidation \textsuperscript{85}. Indeed the in vitro experiments performed in the present study showed that exposure of plasma samples to increased concentration of an oxidant agent resulted in an elevation of all oxidized albumin isoforms. Confirming these results we found a significant positive correlation between all cysteinylated HSA isoforms and the severity of cirrhosis as assessed by the MELD and Child Pugh prognostic scores. Indeed oxidative stress is an hallmark of chronic liver disease and its extent parallels the severity of cirrhosis, being one of the mechanism leading to cell death and organ failure in patient with advanced cirrhosis\textsuperscript{10}.

Higher oxidative modifications to the HSA molecule involving the Cys-34 residue were also found, this was the case of the sulfinylation (HSA-SO\textsubscript{2}H), an irreversible oxidation of the free thiol group on the albumin molecule\textsuperscript{74}. This albumin isoforms is a part of the non-mercaptalbumin-2 (HNA2) fraction, the amount of which was previously found to correlate with the severity of cirrhosis and to predict mortality of patients\textsuperscript{69}. In the present study, although slight increased, the relative amount of the HSA-SO\textsubscript{2}H isoform was not significantly affected by the presence of cirrhosis, however it have to be noted that cirrhotic patients enrolled in our study were characterized by a less severe cirrhosis than patients included in previously reported study, which were mainly characterized by the presence of an acute-on chronic liver failure \textsuperscript{69}. A further oxidative modification of HSA molecule resulted from the formation of albumin dimers. Such alteration was never reported before in patients with liver cirrhosis, and its occurrence was promoted by systemic oxidative stress as suggested by studies performed in other clinical settings\textsuperscript{77}. By performing in vitro experiments we confirmed that oxidative stress represents the main driver for HSA dimers formation. Indeed, following exposure to a pro-oxidant agent, their concentration in plasma samples was found to increase in a time
and concentration dependent manner. We also performed additional experiments in order to better characterize the HSA molecular site involved in dimerization process. The tryptic digestion of plasma samples followed by LC-ESI-MSMS showed that each HSA isoforms resulted from the formation of a disulfide bond between two Cys-34 residue located on two different HSA molecules. Confirming this result the more abundant dimeric HSA isoform we found in plasma samples from both healthy controls and cirrhotic patients were those characterized by a free Cys-34 residue in their monomeric form. Therefore the occurrence of the dimerization process may contribute, with the previously reported oxidative modifications, to a further reduction of the plasmatic free thiol group pool, thus impairing the defense system against oxidative stress. As a matter of the fact all the dimeric isoforms we analyzed significantly correlated with both MELD and Child Pugh scores, being their extent an indirect marker of the increased oxidative stress which characterizes the more advanced phases of cirrhosis.

Glycosylation of the HSA molecule was also found to significantly contribute to the circulating HSA microheterogeneity in both patients and controls. Protein glycosylation is a common alteration promoted by glycemic stress\textsuperscript{86}. This alteration affects both structure and function of the HSA molecule, impairing its binding and detoxification capacity\textsuperscript{67}. The occurrence of protein glycosylation in cirrhotic patients may be expected since about one-third of these patients suffer from diabetes mellitus. Indeed we found that all glycosylated HSA isoforms (HSA+GLYC; HSA+CYS+GLYC) were significantly increased in cirrhotic patients with respect to healthy controls. Further analysis revealed that diabetes is the main driver for the occurrence of the HSA+GLYC isoform, being this specific alteration significantly elevated only in cirrhotic patients with type 2 diabetes mellitus, while the HSA+CYS+GLYC isoform was found to be increased in patients irrespective of the presence of diabetes. Consistently with this observation, the HSA+CYS+GLYC was found to correlate with the MELD score, while an inverse correlation was found between the relative amount of the HSA+GLYC isoform and the severity of cirrhosis. The reason for such result mainly resides in the peculiar distribution of patients with diabetes enrolled in the study among the Child Pugh classes. Indeed diabetic patients were characterized by a less severe cirrhosis in our series.
Finally N- and C-terminal truncated isoforms were also found in plasma samples from cirrhotic patients and healthy controls. The N-terminal portion of the HSA molecule is provided with an indirect antioxidant activity. Indeed, the N-terminal portion of the molecule is able to chelate metal ions, thus contributing to the reduction of reactive species by binding free Cu (I/II) and Fe (II) metals, which are potent enhancer of the production of free radicals. On the contrary an impaired protein stability which leads to a shortening of the HSA circulating half-life was attributed to the truncation of the C-terminal portion of the molecule. While a significant increase of the N-terminal truncated and cysteinylated isoform (HSA-DA+CYS) was found in cirrhotic patients with respect to healthy controls, the relative abundance of HSA isoform characterized only by the truncation of the C- or N-terminal aminoacidic residues did not differ between the two groups, being also slight lower in patients than in controls. The reason for this discrepancy resides in the fact that, these isoforms are characterized by a free Cys-34 residue therefore they may be more susceptible to the dimerization process, and indeed dHSA-DA and d-HSA-L were found to be significantly increased in cirrhotic patients than in controls, being their relative amount directly correlated to the severity of the disease, and, in the case of dHSA-DA, associated to the 1-year mortality.

Another novel finding of the present study is the association existing between specific HSA isoforms and clinical features of cirrhosis, irrespective of the degree of disease severity. Indeed specific pattern of HSA molecular structural alteration were found to be associated to the most frequent complication of cirrhosis, among which ascites, renal impairment and bacterial infection. Monomeric and dimeric HSA isoforms found to be associated to these complications were mainly characterized by a reversible or irreversible oxidation of the Cys-34 residue, thus underling the relevance of systemic oxidative stress in the pathogenesis of event leading to acute decompensation of the disease. On the other hand, it is also reasonable to hypothesize that the pro-oxidant and pro-inflammatory environment occurring during these complications, characterized by increased protease activity, and a storm of pro-inflammatory cytokine and free radical, may favor the multiple structural alterations to the HSA molecule demonstrated in the present study.

As a result of the above described structural abnormalities, the residual amount of the native, unchanged HSA isoform was found to be significantly reduced in patients with
cirrhosis with respect to healthy controls and additionally its relative amount decreased proportionally to the severity of cirrhosis. Being the amount of the residual native HSA profoundly influenced by the wide spectrum of structural alteration occurring in cirrhotic patients carrying different clinical feature, it is not surprising that the native HSA emerged as a better predictor of 1-year survival than total serum albumin concentration, which was an established and widely accepted prognostic marker in the field of chronic liver disease. In this view, while the total serum albumin concentration only reflect the residual functional capacity of the liver, the native HSA encloses information regarding both the liver function and systemic redox state which allow to better predict middle-term prognosis.

The evaluation of the residual functional capacity of the N-terminal portion of the HSA molecule was carried out through the measurement of the circulating level of Ischemia Modified Albumin (IMA). The term Ischemia-Modified Albumin (IMA) identify an HSA isoform with a reduced ability to chelate cobalt at the N-terminal region which is a strong binding site for transition metals, including also copper and nickel. IMA elevation was initially described during myocardial infarction and thereafter during other ischemic conditions, such as stroke or mesenteric ischemia. IMA elevation has been also observed in non-ischemic diseases characterized by enhanced oxidative stress, including ketoacidosis or end-stage kidney disease. Thus, IMA lacks of tissue specificity and reflects a functional disturbance, likely related to a transient conformational change at the N-terminus site as a result of hypoxia, acidosis, or free-radical injury. In the present study we found a significant increase of both absolute IMA levels and IMA to albumin ratio (IMAr) in patients with cirrhosis with respect to healthy controls, however no association were found with the severity of cirrhosis, with exception of an expected correlation between IMAr and Child-Pugh score. These results were partially at variance with previous reports in which circulating IMAr were significantly associated to the MELD score and predict 90-day survival. Besides differences in the sample size between our and the previous mentioned study, it have to be noted that in the study from Jalan et al. patients were screened mainly in intensive care units and were characterized by the presence of acute-on-chronic liver failure, a syndrome associated to a very high mortality rate in the short term.
Given that results it is not surprising the lack of association between circulating IMA and IMAr level and the presence of acute clinical complication of cirrhosis, such as ascites and renal impairment. Contrariwise, an interesting finding is represented by the specific and strong association between circulating IMA and IMAr level and the presence of bacterial infection at hospital admission. Thus the finding of a significant, although moderate, increase in IMA level in the whole population of cirrhotic patients end the rising of both its absolute value and the ratio IMAr following bacterial infection prompt for a role of oxidative stress in the generation of this albumin isoform characterized by an impaired chelating function. Indeed, IMA elevation may occurs in advanced cirrhosis likely as a result of the chronic pro-oxidant and pro-inflammatory state induced by bacterial translocation. In presence of a clinically-evident bacterial infection, which is known to acutely activate the immune response and exacerbate systemic inflammation, a further increase of IMA can be reasonably expected. This hypothesis was also confirmed by the experiment performed in rats with experimental cirrhosis. Indeed, while cirrhotic rats displayed increased IMA level just at baseline, the intravenous administration of a single dose of LPS resulted in a significant rise in IMA level at 6 hours only in rats with cirrhosis. Finally, additional evidences of the relationship between IMA and bacterial infection derive from the multivariate analysis, which showed that IMA level was associated to the infectious complication independently from CRP levels, as well as from the ROC curve analysis according to which the discriminating performance of IMA level was comparable to that of CRP. It have to be noted that the vast majority of the bacterial infection recorded in our population were not characterized by systemic involvement, as diagnosis of sepsis was carried out only in 6 out of 40 patients with bacterial infection at hospital admission. This also explain the fact that procalcitonin levels as well as prognostic scores, such as Child-Pugh and MELD scores, were not significantly increased in patients with bacterial infection with respect to not infected counterparts. Thus, summarizing such results, our data prompt for a potential role of IMA as a new diagnostic biomarker for the early detection of localized bacterial infection.

In conclusion, this study demonstrates that extensive alterations to the HSA molecule, involving both structure and function, occurs in patients with cirrhosis. Structural alteration involves not only the Cys-34 residue, but also other molecular site, being their
extent related to the severity of cirrhosis. Structural alterations were also found to be associated to specific clinical complication of the disease, thus contributing to their poor prognosis. Indeed, the residual amount of the native, unchanged HSA isoform was found to be independently associated to 1-year survival and was characterized by a prognostic power higher than the absolute albumin concentration. In the same patients circulating HSA was found to be also altered at the functional level as suggested by the increased circulating IMA level. Contrary to structural alterations, IMA level was not associated to the severity of cirrhosis nor to the patients prognosis, while a rise in its circulating level was found following acute event, such as bacterial infection, known to enhance the systemic inflammation and oxidative stress.

Overall the findings provided by the present study strengthens the concept of “effective albumin concentration”\textsuperscript{35,95}, which implies that a global assessment of the circulating albumin pool have to take into account not only its absolute concentration, mainly responsible for its oncotic activity, but also its circulating microheterogeneity, and thus its structural and functional integrity, responsible for the non-oncotic activity of the molecule.


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