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DOTTORATO DI RICERCA IN SCIENZE DEI TRAPIANTI

The involvement of the receptor for advanced glycation end products (RAGE)-ligand axis in liver transplantation: hepatic RAGE expression, soluble RAGE and RAGE-ligand levels

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GLOSSARY

AGEs: Advanced Glycation End products

CML: N(epsilon)-Carboxymethyllysine

ELISA: Enzyme-Linked Immunosorbent-Assay

esRAGE: endogenous secreted RAGE

HCC: Hepatocellular Carcinoma

HMGB-1: High-Mobility Group Box 1

HSCs: hepatic stellate cells

LECs: liver sinusoidal endothelial cells

LT: Liver Transplantation

MELD: Model of End-Stage Liver Disease

NAFLD : Non-Alcoholic Fatty Liver Disease

NASH: Non-Alcoholic Steatohepatitis

RAGE: Receptor for AGEs

ROS: reactive oxygen species

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

sRAGE: soluble RAGE

TNF-α: tumor necrosis factor-α

ABSTRACT

BACKGROUND. In animal testing the blockade of the receptor for advanced glycation end products (RAGE) attenuates the liver injury extent induced by RAGE-ligands. Likewise circulating truncated soluble isoforms of RAGE (sRAGE), acting as decoy of RAGE-ligands, protects by injury.

AIM. The primary objective of this study was to investigate the association between tissue RAGE isoform (both full-length and truncated) mRNA expression with both recipient liver disease and early outcomes after liver transplantation. Secondarily, to evaluate trends of circulating RAGE-ligands and of protective sRAGE in the immediate period following transplantation and their association with the development of early organ dysfunction

METHODS. We prospectively included 28 adult LT recipients (53±8.7 years) of primary whole-size grafts by deceased donors (62.1±17.3 years). In liver biopsies of both donor and LT recipients, we measured the transcriptional expression of full-length RAGE and its truncated isoform, the endogenous secreted RAGE (esRAGE). The RAGE-ligands — N(epsilon)-carboxymethyllysine (CML) and high-mobility group protein 1 (HMGB-1) — and the circulating sRAGE were measured in plasma specimens before LT, after graft reperfusion, 1 and 7 days after LT.

RESULTS. In LT recipients the hepatic RAGE mRNA levels were higher than in healthy donors ($p<0.01$). In LT recipients the tissue full-length RAGE inversely correlated with antithrombin III ($\beta= -0.58$, $p = 0.013$) and cholinesterase plasma levels ($\beta= -0.717$, $p= 0.0018$) and directly correlated with MELD score before LT, likewise to basal HMGB-1 plasma levels ($\beta=0.425$, $p=0.043$ and $\beta=0.448$, $p<0.05$ respectively). Basal CML levels were higher in LT recipient than donors ($p=0.02$), decreased after graft reperfusion ($p<0.0001$) but returned progressively to basal values at 7 days after LT. HMGB-1 levels increased after graft reperfusion ($p<0.0001$) and returned suddenly to basal values one day after LT while circulating sRAGE did not change soon after LT but decreased significantly 7 days after LT ($p<0.0001$). The MELD score 7 days after LT inversely correlated with graft esRAGE mRNA expression ($\beta= -0.487$, $p=0.029$) and tended to correlate directly with the peak values of HMGB-1 after reperfusion ($\beta = 0.42$, $p = 0.07$), with recipient age ($\beta = 0.38$, $p = 0.07$) and recipient gender ($\beta = 0.49$, $p = 0.015$). Multivariate analysis showed that, after adjustment for gender, donor age, recipient age, only graft esRAGE mRNA expression was a significant determinant of MELD score 7 days after LT ($\beta= -0.788$, $p=0.0005$).

CONCLUSIONS. The inverse correlation between graft esRAGE mRNA expression and the MELD score 7 days after LT underline the importance of this protective factor for graft survival and patient outcomes. CML accumulation and rapid increase of HMGB-1 followed by remarkable decline of protective sRAGE could have deleterious consequences on graft survival and long term outcomes in LT recipients.

INTRODUCTION

Liver structure, cell types and functions

The liver is the largest gland in the body, consists of two lobes which are wedge-shaped and is situated slightly below the diaphragm and anterior to the stomach. Each lobe is further divided into many small lobules, each being about the size of a pin-head, and consisting of many liver cells, with bile channels and blood channels between them. Two blood vessels enter the liver, namely the hepatic portal vein with dissolved food substances from the small intestine, and the hepatic artery, with oxygenated blood from the lungs. Sinusoids are low pressure vascular channels that receive blood from terminal branches of the hepatic artery and portal vein at the periphery of lobules and deliver it into central veins [1].

The liver, as metabolic center of the body, performs several important functions including control of carbohydrate and lipid metabolism, bile production, red blood cell decomposition, plasma protein synthesis, hormone production, detoxification by harmful substances (e.g. alcohol, drugs and also advanced glycation endproducts, AGEs) [2, 3]. The liver is unique in its ability to undergo compensatory hyperplasia after cell loss by restoring key tissue loss within a few weeks through a complex network of cells and mediators [4].

The liver is composed by different cell types, the parenchymal cells, i.e. hepatocytes, perform the majority of functions including the bile production, while the nonparenchymal cells, represented by three cell types: **liver sinusoidal endothelial cells (LECs)**, **Kupffer cells**, and **hepatic stellate cells (HSCs)** that play different roles **(Fig. 1)**. LECs form the wall of sinusoids and perform important filtration function due to the presence of small fenestrations that allow free diffusion of many substances between the blood and the hepatocyte surface [5]. Kupffer cells are the liver-resident macrophages intrasinusoidally located that modulate the immune function of the liver

and secrete potent mediators of the early inflammatory response like reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α) and other cytokines. Besides typical macrophage activities, Kupffer cells play an important role in the clearance of senescent and damaged erythrocytes [5]. HSCs, located in the perisinusoidal space, store vitamin A, control the turnover of extracellular matrix, and regulate the contractility of sinusoids modulating sinusoidal flow [5]. Acute damage to hepatocytes activates transformation of quiescent HSCs into myofibroblast-like cells that play a key role in the development of inflammatory fibrotic response (see below).

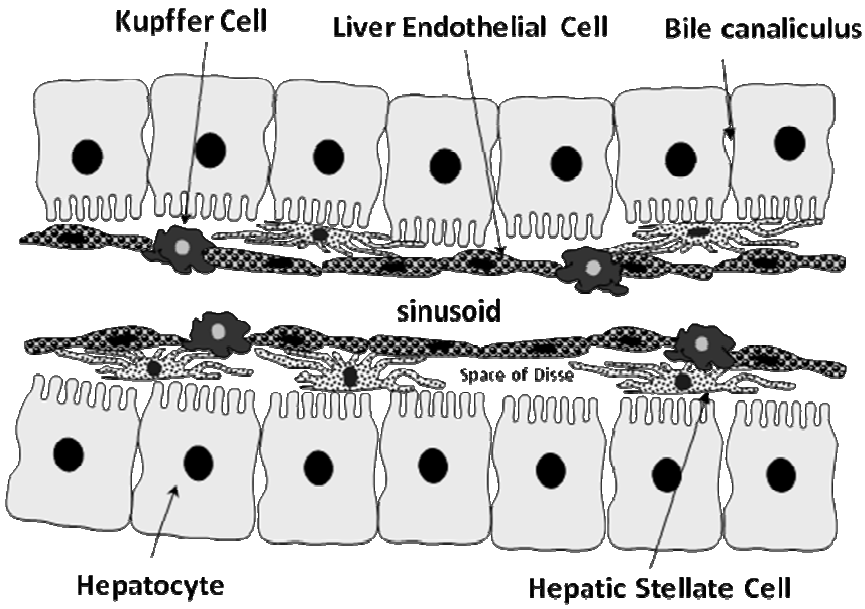

Fig. 1. Cell types organization in liver lobule

Liver transplantation and graft dysfunction

Liver transplantation (LT) represents the lifesaving procedure for patients with chronic end-stage liver disease and acute liver failure (ALF) when there are no alternative treatment options. Incurable chronic liver diseases include liver-based metabolic defect, unresectable hepatocellular carcinoma (HCC), or more commonly, cirrhosis with complications such as hepatic encephalopathy, ascites, hepatorenal syndrome, or bleeding caused by portal hypertension [6, 7].

After allocation to candidates with fulminant hepatic failure, which is fatal within days, the waiting list is sequenced by decreasing model for end-stage liver disease (MELD) score, a very strong predictor of waiting list mortality, that frequently change over time [7]. A recent retrospective survey underscored, among several studied indexes, that the APACHE II death risk scoring system and the **MELD score post-LT, may indicate which patients are at higher risk of early mortality** (up to 30 post-transplantation days) [8].

After liver transplantation, about 25% of recipients develop early allograft dysfunction (EAD), a condition that is associated with significantly decreased graft and patient survival [9]. The concept of early graft dysfunction is not yet clear. There is a spectrum of "graft dysfunction" events: primary nonfunction (PNF), delayed nonfunction, initial poor function (IPF), initial nonfunction, primary graft failure, and primary dysfunction.

A distinction among these entities considers the grade of dysfunction, the need for urgent retransplantation, and their timing and length after LT [10]. According to the United Network for Organ Sharing (UNOS), primary dysfunction is defined as unrecoverable graft function needing urgent liver replacement during the first 10 days after LT. It is characterized by an aspartate amino transferase (AST) $\geq$ 5000 UI/L, an international normalized ratio of prothrombin (INR) $\geq$ 3.0, and acidosis (pH $\leq$ 7.3

and/or lactate concentration ≥ 2× normal) [10]. The factors that may impact the occurrence of EAD could be: donor-related factors, including advanced donor age and steatotic graft, and recipient-related factors, including higher MELD scores, septic complications, rejection and biliary complications, among which the age of the donor and MELD score represented the best predictive factors for EAD onset [9, 11].

Cellular mechanisms of graft dysfunction: ischemia/reperfusion (I/R) injury

Ischemia/reperfusion (I/R) injury results from a prolonged ischemic insult followed by restoration of blood perfusion, and it is a pivotal phase in transplantation, and in particular in LT, in which the damage is sustained during cold preservation of the liver following explantation from the donor, and during subsequent warm reperfusion at implantation into the recipient [12]. The development of EAD is often thought to be secondary to substantial I/R injury, which is associated with acute cellular damage, cell death, oxidative damage from the creation of ROS, and a severe inflammatory response occurring within the liver [9, 13]. The degree of I/R injury sustained is dependent on the length and method of ischemia applied to the liver, as well as the background liver condition. Moreover, patients who undergo short periods of ischemia sustain less liver dysfunction compared to those undergoing a long period of ischemia [14].

Liver I/R injury is a complex process involving numerous cell types among which Kupffer cells play a key role in initiating and propagating cellular damage and death in I/R injury, they are activated during the ischemic phase and even more so on reperfusion [12]. During the ischemic phase, as a result of glycogen consumption and lack of oxygen supply, Kupffer cells, SECs, and hepatocytes suffer with lack of adenosine triphosphate (ATP) production. This lack leads to dysfunction in homeostatic processes that carry out edema and swelling. Kupffer cells and LECs swelling, combined with an increase in the vasoconstrictors endothelin and thromboxane A_2 due to the reoxygenation, and a decrease in the vasodilator nitric oxide, lead to activation of HSCs and constricting hepatic sinusoids. The end result is the significant reduction of microcirculatory blood flow on reperfusion, including some areas with complete absence of blood flow, which is known as “no-reflow” [15].

One of the principal actors involved in the I/R dangerous processes, and that is released in response to hypoxia, is the nuclear transcription factor **high mobility group box-1 (HMGB-1)**. HMGB-1 is a DNA-binding protein present in the nucleus of almost all eukaryotic cells which can be either released passively by necrotic hepatocytes or the damaged extracellular matrix, or is actively secreted, following cytokine stimulation, by stressed and injured liver cells, so its levels are significantly elevated in the serum and liver tissue after I/R, and it can lead to prolonged liver injury [16]. HMGB-1 exert its effects by binding to a group of receptors among which there are the toll-like receptors (TLRs), that include the **receptor for advanced glycation end products (RAGE) (Fig. 2)**.

HMGB-1 and other mediators released by necrotic hepatic cells represent the inflammatory signal transduction mediators that cause activation of hepatocytes and Kupffer cells leading to the activation of the nuclear factor kappa B (NF-κB) pathway [17]. This activation leads to the expression of many cytokines and chemokines, including TNF-α, interleukin-1b (IL-1b), IL-6 and IL-8 that in turn induce expression of many other inflammatory mediators in injured liver tissue [18, 19]. TNF-α is the central component of the proinflammatory cytokine cascade in liver I/R injury and acts both locally in a paracrine fashion, and remotely as an endocrine mediator. The up-regulation of TNF-α leads to liver damage by binding to specific receptors on the surface of hepatocytes and this results in increased production of ROS, and further activation of NF-κB [19].

ROS have widespread effects in the initiation and propagation of I/R injury. During oxidative stress, localized expression of monocyte chemotactic protein-1 (MCP-1) attracts monocytes and activated lymphocytes to sites of injury. Likewise, IL-8 causes sinusoidal neutrophil sequestration and resultant hepatocellular damage [18, 19].

Moreover ROS can activate SECs, leading them to NF-κB mediated expression of cell-surface adhesion molecules (i.e. intercellular adhesion molecule-1, ICAM-1 and vascular adhesion molecule-1, VCAM-1), that lead to further monocytes adhesion to I/R injured site [20]. Therefore, excessive activation of the NF-κB pathway may be detrimental to early liver allograft function post-transplantation. A link between NF-κB regulation and EAD was anticipated because induction of the NF-κB-associated genes in Kupffer cells is an early event following I/R injury, and serves as a point of convergence for molecular signals inherent in many forms of chronic liver disease. Actually, EAD is associated with early post-operative increases in MCP-1, IL-8, and IL-6 whose expression is driven by NF-κB activation [21].

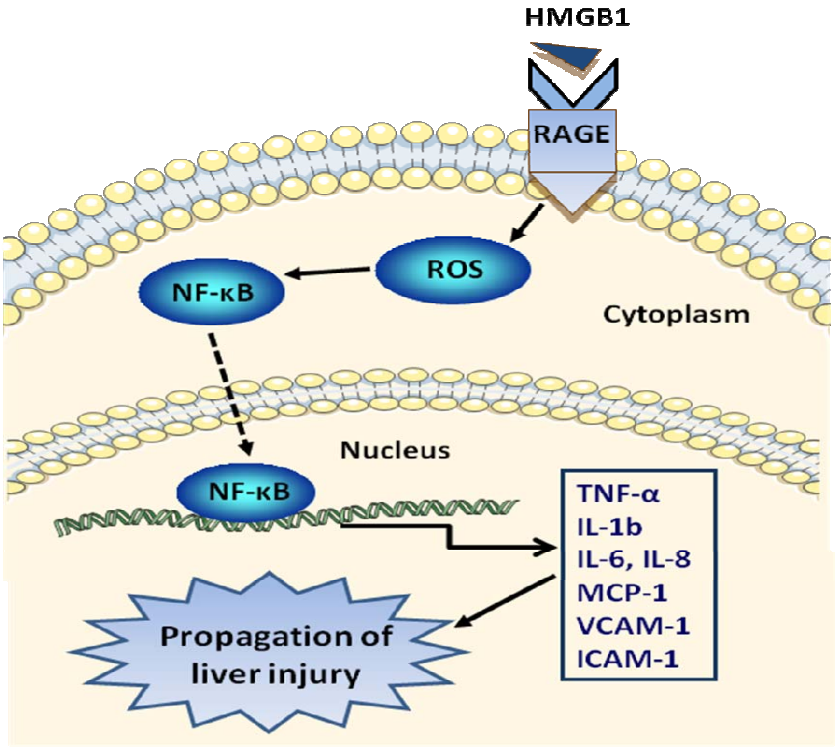

Fig. 2. The RAGE-HMGB-1 interaction mediated signal transduction pathway in liver cells

RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS (RAGE)

Structure of RAGE and soluble forms of RAGE: the cleaved-form and splicing variant esRAGE

The RAGE is a multiligand-binding member of the immunoglobulin superfamily of cell-surface molecules [22]. The full length receptor consists of an extracellular region formed by one V-type immunoglobulin domain, needed for ligand binding, and two C-type immunoglobulin domains, followed by a single, short transmembrane domain and a short cytoplasmic domain, that is essential for RAGE-mediated signal transduction [22, 23] **(Fig. 3)**. RAGE is expressed on many cells including endothelial cells, mononuclear phagocytes, lymphocytes, vascular smooth muscle cells, neurons and some cell types of the liver [6, 22, 24]. The RAGE is typically expressed at low levels under normal physiological conditions in the majority of tissues and organs, except in the lung where exhibits high basal levels of expression. High levels of RAGE expression occur in the other tissues only under pathological conditions [25-28]. The biological activity of RAGE is dependent on its ligation by various ligands, released by inflamed, stressed and damaged cells [29].

Its name is derived from the first-known ligands, the **advanced glycation endproducts (AGEs)**, a complex and heterogeneous group of tissue-bound and circulating glycoxidated proteins, of which the **carboxymethyl-lysine adducts (CML)** are the most abundant [30]. Apart the AGEs, that are occasional ligands, the RAGE binds the non-histone nuclear factor **high-mobility group box 1 protein (HMGB-1)**, certain members of the S100/calgranulin proinflammatory cytokine family, the β-amyloid peptide, the β-sheet fibrils and others [31-34]. Increased expression of both cell-surface RAGE and accumulation of its ligands are observed in a range of disorders

characterized by chronic inflammation, such as inflammatory bowel disease, rheumatoid arthritis, atherosclerosis, amyloidoses, tumors, Alzheimer's disease, the vascular complications of diabetes and in liver impairment [35, 36].

Besides the full-length receptor, several truncated isoforms of the RAGE have been described, some of which are soluble isoforms containing only the extracellular portion of the full-length receptor and that are detectable in circulating blood (reviewed in Ref. [37]). Two major RAGE mRNA splice variants have been thoroughly characterized. One variant protein (N-truncated type) lacks the V-type immunoglobulin domain, but it is otherwise identical to full-length RAGE and is retained in the plasma membrane [38] **(Fig. 3)**. As a result of the deletion of the V-type immunoglobulin domain, this variant protein is significantly impaired in its ability to bind RAGE ligands [38]. The other variant (C-truncated type), named **endogenous secreted isoform (esRAGE),** containing the same immunoglobulin domains present in full-length RAGE, but lacking the cytosolic and transmembrane domains [38], is secreted extracellularly and can be detected in circulating blood.

Total circulating isoforms of RAGE, overall named **soluble RAGE (sRAGE),** include both esRAGE and a truncated form generated through proteolytic cleavage of the full-length cell-surface receptor, probably mediated by membrane metallopeptidases, including ADAM10 [39] **(Fig. 3)**. Both esRAGE and the cleaved-type of soluble RAGE are still able to bind ligands and, by competing with membrane-bound RAGE for ligand binding [38, 40], can have cytoprotective effects [38, 41]. Higher circulating levels of sRAGE are associated with reduced risk of coronary artery disease (CAD), hypertension, metabolic syndrome, and other chronic diseases [42-45].

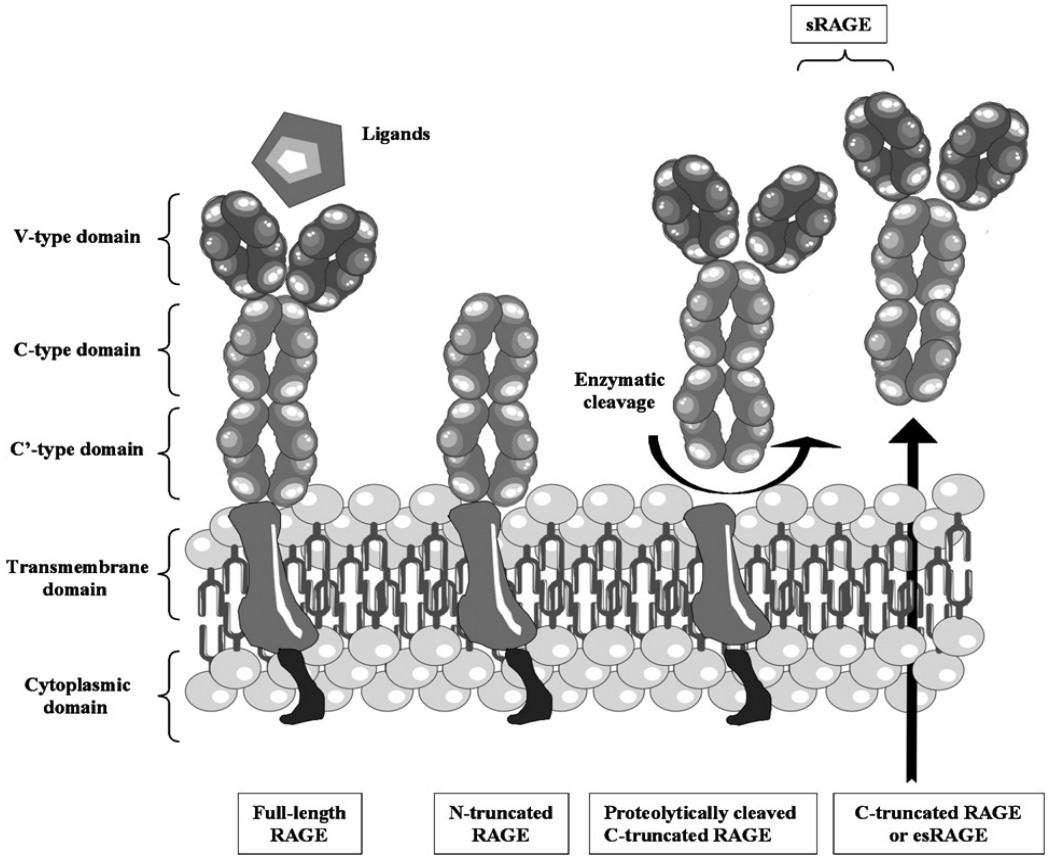

Fig. 3. The structure of RAGE and its soluble forms.

Expression of RAGE and esRAGE in liver tissue

In literature, conflicting evidences about the RAGE expression and localization among the different cell types of the liver and across different species, have been reported. The first evidence of the expression of this receptor in normal liver tissue, dates back to 1993 when in bovine liver the RAGE was found in hepatocytes, whereas no significant expression was detected in Kupffer cells and LECs [25]. More recently, Butscheid et al. [46] reported marked levels of RAGE in hepatocytes and in bile ducts of healthy human liver and a weak staining for RAGE in Kupffer cells.

Conversely, another study later revealed that RAGE was exclusively expressed by HSCs isolated from rat liver [47], whereas no transcripts were seen in hepatocytes, Kupffer cells, or LECs isolated in both rat and mouse liver [3, 47]. Another study performed on human HSC lines highlighted the expression of RAGE at both transcript and protein levels [48]. These results have been confirmed in rat and human HSC lines that expressed significant RAGE while no RAGE expression was found in rat hepatocytes [49]. The discrepancies among RAGE expression patterns described by different research groups may be due to dissimilar experimental settings that included the use of antibodies with different specificities.

However, summarizing these studies it may be essentially said that there is a cell specific pattern of RAGE expression and there is enough agreement that the RAGE is poorly or not expressed in Kupffer cells and LECs in almost all species analyzed, while it is markedly expressed in HSCs and in hepatocytes [6].

INVOLVEMENT OF RAGE-LIGAND AXIS IN LIVER DISEASES

The catabolism of AGEs: lack of RAGE involvement

RAGE is associated with many inflammation-related pathological states [36, 50], but there is no evidence that it plays a catabolic function. On the other hand, in the liver such a function is carried out by other AGE-receptors that act mainly as scavenger receptors for AGE detoxification and catabolism [3, 46, 51]. This has been shown in an in vivo study in rats demonstrating that the liver removed from the circulation more than 90% of intravenously injected AGEs via endocytosis mediated by scavenger receptors in LECs (60%) and in Kupffer cells (25%), whereas the contribution of hepatocytes was low (10-15%) [52]. These results were later confirmed in cultured LECs incubated with AGEs and whose intracellular uptake was mediated by scavenger receptors [52]. Furthermore, experiments utilizing peritoneal macrophages and LECs derived from macrophage class-A scavenger receptors (MSR-A) knock-out mice, showed that in peritoneal macrophages AGEs were endocytosed almost exclusively through MSR-A, whereas in LECs the uptake of AGEs took place through a pathway distinct from MSR-A [3, 53]. Another study performed on biopsy specimens from patients with varying degrees of hepatic dysfunction revealed that, irrespective of diagnosis, CML and galectin-3 (another AGE-scavenger receptor), but not RAGE, were highly expressed in Kupffer cells [46]. Therefore, AGE catabolism does not seem to be mediated by RAGE for three reasons: first, expression of RAGE in hepatic cells involved in AGE-endocytosis (mainly LECs and Kupffer cells) is null or weak; second, RAGE is functionally similar to a cell signaling receptor rather than a scavenger receptor, and thirdly scavenger receptors (i.e., galectin 3 and MSR-A) for AGEs also exist in the liver [3, 46]. Since the liver is the major site of AGE catabolism, whatever

the scavenger receptors involved in endocytosis of AGEs may be, a consequence of impaired hepatic function is the increase of circulating AGEs levels, which can exert their detrimental effects on the whole organism.

Pre-clinical studies: RAGE involvement in liver fibrosis

The first study on the role of RAGE in hepatic injury, was carried out in a mouse model of total hepatic ischemia/reperfusion (I/R) in which the blockade of the RAGE, through administration of sRAGE, which functions as a ligand-competitor, protected against the hepatocellular necrosis, attenuated liver I/R injury and enhanced the expression of the proregenerative cytokine TNF-α [54]. Recently, in the same model, it has been shown that early growth response-1 (Egr-1), an inducible transcription factor activated by stress stimuli, was upregulated in the liver remnants after hepatic I/R injury and it was suppressed by administration of sRAGE or in RAGE knock-out mice [55]. Moreover, the RAGE-ligand HMGB-1, released from necrotic cells or following induced hypoxia, was upregulated after I/R in the liver remnants and it can limit liver regeneration and response to I/R injury [16, 55].

So, the activation of RAGE may contribute to induction of pro-inflammatory and tissue-destructive processes on hepatic I/R, and the blockade of RAGE may limit immediate deleterious inflammatory mechanisms, and thereby facilitate regenerative potential in the injured liver offering a potential target in clinical transplantation.

In a mouse model of hepatectomy, RAGE was up-regulated in liver remnants after massive versus partial hepatectomy, principally in monocyte-derived dendritic cells [56]. Blockade of RAGE, using pharmacological antagonists or transgenic mice in which a signaling-deficient RAGE mutant is expressed in cells of monocyte lineage, significantly increased survival of the mice after massive liver resection and increased proliferation and reduced apoptosis of hepatocytes in liver remnants [56]. Furthermore, liver remnants retrieved from RAGE-blocked mice displayed enhanced expression of TNF-α and IL-6, cytokines promoting inflammation and regeneration and of the anti-inflammatory IL-10, suggesting that the RAGE mediates injurious stress responses in

liver resection and initiates events that critically curtail the limits of regeneration while the RAGE blockade may be a novel strategy to promote regeneration in the massively injured liver [56].

The active involvement of RAGE in the liver was shown also in an acetaminophen-induced hepatotoxicity in a mouse model in which the treatment with sRAGE increased the animal survival, attenuated the hepatotoxicity, decreased the hepatic necrosis and enhanced the expression of TNF-α and IL-6 [57].

Excluding the lung in which physiological high levels of RAGE expression seem to have a protective role in preventing pulmonary fibrotic disease, high expression levels of RAGE seem be instead associated to renal and liver fibrosis [49, 58, 59].

A number of studies have reported that the RAGE expression is up-regulated during the trans-differentiation of HSCs to myofibroblasts, their spreading and migration [47, 60, 61].

As previously described, after hepatic I/R injury, there is an inflammatory milieu and HMGB-1 is actively and passively released by the damage liver cells [62] and, through RAGE engagement, it can directly activate HSCs and drives them toward fibrogenesis, stimulating cell proliferation and alpha-smooth muscle cells (α-SMA) gene and protein expression and suppressing MMP-2 activity **(Fig 4)**. This process seems not to be mediated by CML or AGEs [49]. In addition, in rats in which liver fibrosis was induced by bile duct ligation or through thioacetamide treatment, the RAGE expression and the α-SMA transcript, were up-regulated in the cirrhotic liver [49].

Recently, in the same animal model, AGEs administration significantly increased collagen content and α-SMA expression, that are markers of hepatic fibrosis, compared with bile duct ligation alone. Moreover, AGEs increased hepatic oxidative stress and RAGE gene expression, suggesting that AGEs may contribute to the pathogenesis of

chronic liver injury and fibrosis [63]. In another rat model in which hepatic fibrosis at different stages was induced by carbon tetrachloride, the gene silencing of RAGE suppressed NF-κB activity, HSCs activation, and accumulation of extracellular matrix proteins in the fibrotic liver, improving the ultra-structure of liver cells [59]. Altogether these observations suggest that the RAGE may be implicated in the initial processes of liver fibrosis and that the RAGE-blockade or the RAGE-gene silencing may be a therapeutic modality to prevent fibrogenesis in post-transplant liver grafts.

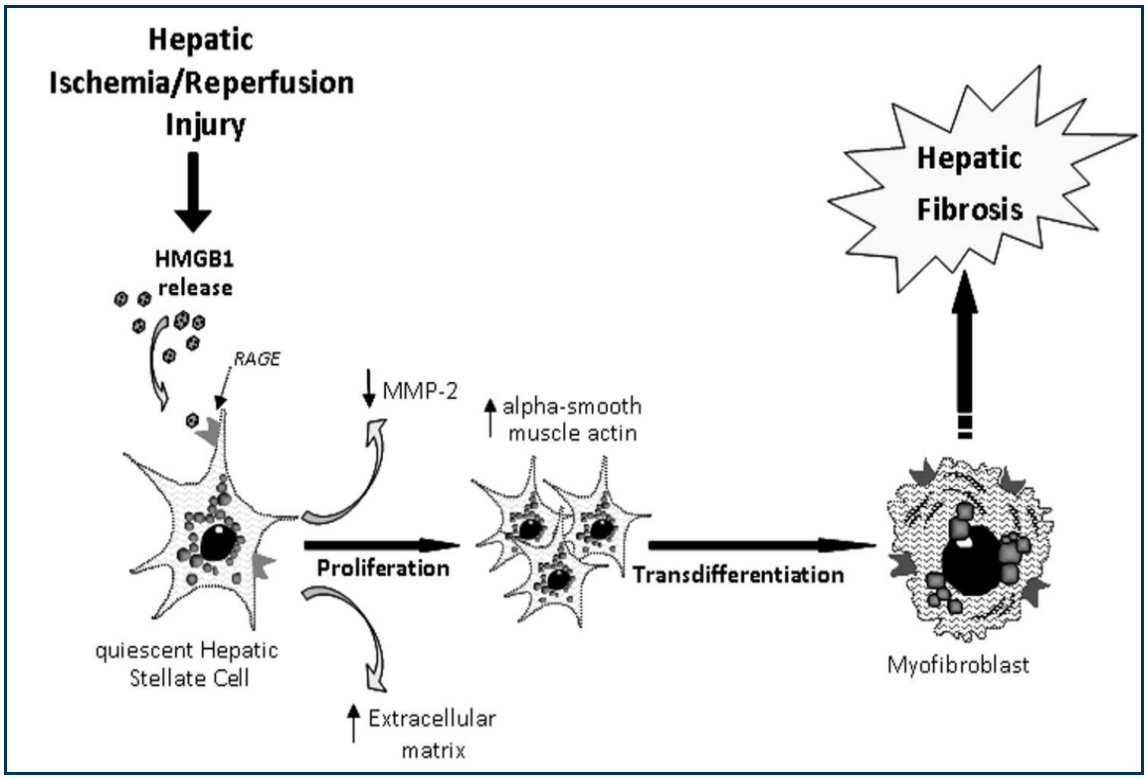

Fig. 4. The role of RAGE in hepatic fibrosis

Clinical association studies

The liver being a multifunctional organ (specialized in detoxification, metabolism and defence), is also prone to many diseases including non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Histologically indistinguishable from alcoholic liver disease, the exact etiology of NAFLD remains unknown, but the fundamental pathophysiological process appears to be insulin resistance and oxidative stress related to the metabolic syndrome [64], and NASH is the more severe form of NAFLD.

Several clinical studies have suggested the involvement of ligands (AGEs, CML, HMGB-1) and RAGE (tissue-bound and soluble form) in different liver diseases such as NASH, liver cirrhosis and HCC.

Butscheid et al. [65] have not found any difference in CML levels among patients with hepatitis C virus (with or without fatty liver), with NAFLD or with NASH. Conversely, in patients with liver cirrhosis, high levels of circulating CML correlated positively with the severity of the disease and inversely with residual liver function [66]. Further, serum CML levels that were significantly higher in patients with chronic liver diseases than healthy controls, were found closely associated with liver function capacity [67]. Although apparently discordant, taken together these results suggest that a moderate impairment in hepatic function does not affect circulating CML levels that instead increase in severe cirrhosis as consequence of a reduced AGE-catabolism rather than as cause of the disorder.

In a recent study serum levels of HMGB-1 were found significantly higher in patients with chronic hepatitis B virus and with low fibrosis compared to controls [68].

The levels of circulating sRAGE, the cell-membrane RAGE competitor for ligand binding, were found significantly lower in patients with NASH but not in patients with

simple fatty liver compared to controls [69]. Recently, in obese pre-pubertal children with and without liver steatosis, esRAGE and sRAGE levels were found significantly lower in those affected by liver steatosis and were independently related to liver steatosis, suggesting that the ligand-RAGE pathway plays an independent role in the development of liver injury, already in this age group [70].

Cheng et al. [28], using a tissue microarray technique, have studied the expression profile of esRAGE in human organs highlighting the distribution of esRAGE as dot-like granules in the supranuclear regions facing the luminal surface of the bile ducts; esRAGE expression decreased in hepatocytes of patients with obstructive jaundice suggesting that esRAGE may contribute to secretion of bile [28].

A number of studies have compared different patterns of expression of RAGE, esRAGE and ligands in liver specimens among subjects with varying degrees of liver impairment. Patients with NASH, but not subjects with simple steatosis, had detectable tissue AGEs in hepatocytes [71]. In a study of immunostainings of biopsy specimens from subjects of different diagnoses (healthy control, steato-hepatitis, virus related hepatitis, cholestasis and cirrhosis), CML and RAGE were detected in hepatocytes of all patients, independently of diagnosis [46].

Liver tissues from normal, hepatitis and HCC subjects showed co-expression of RAGE and HMGB-1 transcripts in all subjects [72]. However, whereas the HMGB-1 transcripts were comparable with each other, the level of RAGE was different, i.e., lower in normal liver compared to hepatitis and highest in HCC [72]. These data suggest that RAGE expression is particularly significant in liver of patients with HCC, probably because this receptor upregulates the transcription of its own gene through a positive feedback loop with its ligands that are produced abundantly during the HCC

development (e.g. HMGB-1 may be released from necrotic cells and AGEs generated following altered hepatic glycaemic control).

Over the last decade, data about involvement of the RAGE-ligand axis in inflammation and regeneration after solid organ transplantation has emerged from clinical studies on the lung [73-75], kidney [73, 74], heart [76], and LT [66, 77, 78]. Interestingly, elevated plasma CML levels in patients with liver cirrhosis decreased by 50% three months after LT confirming that the liver acts as a clearing organ for AGEs through AGE scavenger receptors [66].

More importantly, during LT circulating levels of the pro-inflammatory RAGE ligand HMGB-1 were undetectable before graft reperfusion and increased after reperfusion, and their level was correlated to graft steatosis [77]. These data suggest that HMGB-1 originates from the graft and may be a marker of hepatocellular injury.

AIM OF THE STUDY

During the last few years the scientific interest about the implications of RAGE and its ligands in liver impairment has grown up and there are many indications, although not unequivocal, about a potential role of RAGE in liver diseases as well as the existence of a protective role of sRAGE against the hepatocellular injury. From in vitro and experimental studies emerged that the blockade of RAGE attenuated the liver injury extent induced by RAGE-ligands. Therefore, the RAGE-ligand axis, through the activation of intracellular signals and the resulting production of cytokines, may be implicated in the processes of liver inflammation. The primary objective of this study was to investigate the association between tissue RAGE isoform (both full-length and truncated) mRNA expression with both recipient liver disease and early outcomes after liver transplantation. Secondarily, to evaluate trends and changes of circulating RAGE-ligands and of protective sRAGE in the immediate period following transplantation and their association with the development of early organ dysfunction.

MATERIAL AND METHODS

Material

Bovine serum albumin (BSA), agarose and RNA*later* (Sigma-Aldrich, St Louis, MO), 96 wells plate (Nunc-Immuno Plates Maxisorp, Denmark), anti-AGE-peroxidase conjugated monoclonal antibody (clone 6D12) (Cosmo Bio Co., LTD, Tokyo, Japan), ELISA kit for sRAGE (DuoSet ELISA Development kit, R&D systems, Minneapolis, MN), tetramethybenzidine (TMB) (Sigma, St. Louis, MO), ELISA kit for HMGB-1 (double-sandwich ELISA Kit II, IBL International, Hamburg, Germany), RNeasy Midi kit, QIAzol lysis reagent and Taq PCR Core Kit (Qiagen S.p.A, Milan, Italy), iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc, Hercules, CA, USA), PCR primers (Eurofins MWG Operon), BioPhotometer (Eppendorf Italia, Milan, Italy). **Buffers:** Coating buffer for CML (50 mmol/L baking soda, pH 9.6), coating buffer for sRAGE (10 mmol/L PBS, pH 7.2), blocking/reagent buffer (PBS with 1% BSA), washing buffer (PBS with 0.05% Tween 20).

Patients enrollment

We prospectively enrolled twenty eight consecutive patients with end stage liver diseases undergoing primarily liver transplantation (LT) at the General Surgery and Liver Transplantation Unit of Pisa between November 2010 and April 2012. We excluded from the study subjects with at least one of the following conditions: chronic kidney disease (serum creatinine values of 1.5 mg/dL or greater), prior heart failure, multiple organ graft recipients, inability to provide informed consent to the study. Each person provided their medical history, underwent clinical examination, and abdominal TAC. MELD score (Mayo End stage Liver Disease) of the patients was calculated according with the original version of the MELD scale as developed by investigators at Mayo Clinic. All subjects gave written informed consent for their participation in the

study which was approved by the IRB (Institutional Review Board) and was conforms to the principles outlined in the Declaration of Helsinki.

Liver biopsies and blood samples collection

Two biopsies were obtained respectively from graft during liver procurement and from the explanted liver of the recipients, and were stored at -20°C in RNA*later* to stabilize the tissues. A single systemic blood sample was drawn from donors and from recipients immediately before surgery. During LT, the timing of blood samples collection was within 30 minutes after graft reperfusion, 1, 7 and 30 days after LT. Blood samples were collected in tubes containing K_3EDTA, centrifuged at 4°C and plasma samples were immediately stored at -80°C until analysis. All laboratory work was performed in blinded fashion with respect to the identity of the samples. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), bilirubin, plasma international normalized ratio (INR), Antithrombin III and cholinesterase, were determined by standard laboratory methods.

Enzyme-linked immunosorbent-assay (ELISA): determination of sRAGE, CML and HMGB-1 plasma levels

Plasma sRAGE levels were determined using a double-sandwich ELISA kit as previously described [79]. Briefly, 96-well microplates were coated with monoclonal antihuman RAGE (1 μg/mL) in coating buffer and incubated overnight at room temperature (RT). Four rinses with washing buffer followed each incubation step. After blocking with reagent buffer at RT for 1 h, 100 μL of sample diluted in reagent diluent was added and incubated at RT for 2 h. Recombinant human RAGE was used as standard in a concentration range of 31.2–4000 pg/ml. Then 100 μL of biotinylated goat

antihuman RAGE (200 ng/ml) was added and incubated at RT for 2 h. Next, 100 µL of streptavidin-horseradish peroxidase (1:200) was added and incubated for 20 min at RT. Finally, 100 µL of tetramethybenzidine (TMB) substrate was added. After 5–30 min, 50 µL of 2 mol/L sulfuric acid was added to stop the reaction and OD at 450 nm determined by an ELISA plate reader. The intra-assay and inter-assay coefficients of variation were less than 5.9% and less than 8.2%, respectively. The lower limit of detection of sRAGE was 21.5 pg/ml.

Plasma CML levels were measured by an in-house competitive ELISA using the mouse F(ab')2 fragment anti-AGE monoclonal antibody as previously described [30]. Briefly, CML–BSA in coating buffer was coated for 48h at 4°C on 96-well ELISA plates. Wells were washed three times with washing buffer, and then blocked with 100µl blocking buffer (PBS containing 1% BSA) at room temperature for 1h. After three rinses with washing buffer, 50µL of diluted sample were added, followed by 50µl of anti-AGE antibody-peroxidase conjugate (0.1µg/mL) in blocking buffer. Chemically synthesized CML–BSA was used as standard in a concentration range of 0.1–100µg/mL. Plates were incubated at room temperature for 3h with gentle agitation on a horizontal rotary shaker. After three rinses, 100µl of TMB was then added to each well. After 5 to 30min, 50µL of 2 mol/L sulphuric acid was added to stop the reaction and the optical density at 450nm determined by an ELISA plate reader. Intra- and inter-assay coefficients of variation of our assay were 3.2 and 8.7%, respectively. The lower limit of detection of CML was 0.5µg/ml.

Plasma HMGB-1 levels were determined using the double-sandwich ELISA Kit II according to the manufacturers' description. Briefly, 50 µL/well of diluent buffer and 50 µL of plasma samples, standard or positive control were added at a 96-well microplates, precoated with polyclonal antibody specific for HMGB-1, and incubated

for 20-24 h at 37°C. Recombinant human HMGB-1 was used as standard in a concentration range of 40-1.25 ng/ml. Five rinses with washing buffer followed each incubation step. After, 100 µL of POD-conjugate anti-HMGB-1,2 monoclonal antibody were added and incubated for 2 h at 25°C. Then, 100 µL of colour solution were added to the wells and incubated for 30 min. Thereafter, 100 µL of stop solution were added and OD at 450 nm was determined by an ELISA plate reader. Intra-assay and inter-assay coefficients of variation values were <10%. The sensitivity of the assay was 0.2 ng/mL.

Total RNA extraction

Total RNA was extracted from liver specimens as previously described [80] but using the RNeasy Midi kit in order to obtain a larger amount of purified RNA. Briefly, tissue samples were cut on a sterile plate, weighed and break into pieces. One mL of QIAzol lysis reagent was added for each 100 mg of tissue. A stainless steel bead was added to the tube and the tissues were homogenized for 5 min at 30 Hz by TissueLyser. The homogenate was centrifuged at 10000 g for 10 min at 4°C to remove the insoluble material, and the supernatant was transferred in a new tube in which 1 volume of 70% ethanol was added. The tube was mixed, then the sample was applied to an Rneasy Midi column and processed according to the manufacturers' description. The RNA concentration was determined spectrophotometrically at 260 nm. The integrity and purity of total RNA were also detected by electrophoresis of samples on ethidium bromide 2% agarose gel and visualized at 302 nm (2UV^{TM} Transilluminator, UVP, Upland, CA). The RNA samples were stored at -80°C and used for gene expression studies.

Semiquantitative detection of mRNA by RT-PCR

The reverse transcription of total RNA to cDNA was carried out using iScript cDNA Synthesis kit and for template amplification, the Taq PCR Core Kit was used in a GeneAmp PCR System 9700 thermalcycler (Pelkin Elmer, Lockport Place, Lorton) according to the manufacturers' description.

The PCR conditions used for human RAGE (isoform_1) (GenBank ID: NM_001136.4) were the follow: denaturation at 94°C for 4 min followed by 40 cycles, in which each cycle consisted of denaturation at 94°C for 40 sec, annealing at 62°C for 30 sec, elongation at 72°C for 50 sec, and final extension at 72°C for 7 min. The primers used for RAGE (201 base pair) were: forward 5'-CAG GAA TGG AAA GGA GAC CA-3' and reverse 5'-CCC TTC TCA TTA GGC ACC AG-3' [81]. The PCR conditions used for esRAGE (isoform_6 and 9) (NM_001206940.1 and NM_001206966 respectively) were the follow: 95°C for 90 sec followed by 40 cycles of 95°C for 5 sec, 55°C for 15 sec and 70°C for 25 sec. The primer used for esRAGE (89 bp) were: forward 5'-GGG GAT GGT CAA CAA GAA AGG-3' and reverse 5'-AGG TTC CTC CGA CTG ATT CAG TTC-3'. The RAGE and esRAGE mRNA expression were normalized with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a constitutive human gene used as an internal control. The primers and the PCR reaction steps used for GAPDH (354 bp) were: forward 5′-GGT CTC CTC TGA CTT CAA CAG CG-3′ and reverse 5′-GGT ACT TTA TTG ATG GTA CAT GAC-3′. The PCR condition used for GAPDH were the follows: 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 68°C for 7 min [82]. All primers were analyzed using Primer BLAST against GenBank database to determine the identity of the sequences and the length of the relative PCR products. The amplified products

were electrophoresed on ethidium bromide 2% agarose gel, in parallel with a DNA Ladder 100 bp and visualized with an ultraviolet transilluminator.

Statistical analysis

Sample size was calculated with the Stata software (version 9.2; Stata Corp. College Station, Texas, USA) by the estimated power for two-sample comparison of means of graft esRAGE mRNA values. A sample size of 56 patients (28 per arm) would provide 85% power to detect differences of 30% in esRAGE value between two groups in a two-sided test at an α level of 0.05. Data were analyzed with the use of statistical software SPSS 13.0 (SPSS Inc, Chicago, Ill, USA).

The Kolmogorov–Smirnov test of normality was used to verify whether the distribution of variables followed a Gaussian pattern. Data with a normal distribution are given as mean±SD. Variables with a skewed distribution are expressed as median and interquartile range. Group differences were analyzed by Student t-test, and $\chi 2$ test for normally distributed, and non-continuous variables, respectively. Variables with a non-normal distribution were logarithmically transformed before each analysis.

Multiple differences were evaluated by one way ANOVA followed by Bonferroni post hoc test. A multivariate regression analysis, adjusted for donor age, recipient age and gender, was used to identify the significant factors associated with the MELD score at 7 days after-LT. A two-tailed p-value < 0.05 was considered statistically significant.

RESULTS

The biochemical and demographic characteristics of donors and recipients (n= 28) before surgery and in the first 7 days after surgery are given in **Table 1**.

Table 1. Donors and recipients characteristics

Variables		
Donors		
Age (years)	62.1 ± 17.3	
Recipients		
Male (%)	23 (82%)	
Age (years)	53 ± 8.7	
Primary diagnosis, n (%)		
Hepatocellular Carcinoma (HCC)	7 (25%)	
HCC +HCV+ cirrhosis	7(25%)	
HCC +HBV+ cirrhosis	2(7.1%)	
HCV+ cirrhosis	5(17.9%)	
HCC + HBV	1 (3.6%)	
HCC + HCV	1 (3.6%)	
Caroli's syndrome	2(7.1%)	
Primary sclerosing cholangitis	2(7.1%)	
Alcoholic cirrhosis	1 (3.6%)	
	Before LT	**On day 7**
Alanine Aminotransferase (ALT) (U/L)	69 (42-90.5)	89 (76.2-119.8)
Aspartate Aminotransferase (AST) (U/L)	72.5 (59-134.5)	40 (31.2-56.8)
Gamma-glutamyl transpeptidase (GGT) (U/L)	141.5 (104-197.5)	159 (106-220)
Lactate Dehydrogenase (LDH) (U/L)	232.7 ± 59.1	234.8 ± 39.4
Bilirubin (mg/dL)	1.51 (1.1-2.5)	3.28 (1.8-4.4)
MELD score	9.0 (6.0-13)	9.97(7.7-13.4)
Cholinesterase (U/L)	2663 (2049-5335)	
Antithrombin III (%)	50 (38-73)	
Ascites, n (%)	10 (36%)	
Graft cold ischemia time (minutes)	451.4 ± 91	
Graft warm ischemia time (minutes)	87 ± 15	

Data are presented as mean ±SD, medians and interquartile ranges or percentage.

As can be inferred from the table, most of our patients had HCC (64%), were male (82%) and with an average age lower than donors (53 ± 8.7 versus 62.1 ± 17.3 years).

In **Fig. 1** we showed an example of gel electrophoresis for PCR products of RAGE (201 bp) in donors and recipients.

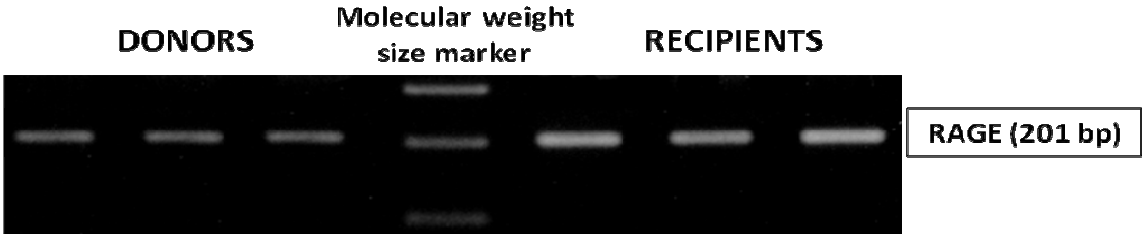

Fig. 1. An example of gel electrophoresis of PCR products of RAGE in donors and recipients

From RT-PCR analysis of hepatic full-length RAGE expression emerged that LT recipients had higher transcriptional levels of tissueRAGE than donors (972 ± 148 *versus* 831 ± 191 mU, $p < 0.01$).

The linear regression analysis highlighted that recipient hepatic RAGE mRNA expression inversely and significantly correlated with antithrombin III ($\beta = -0.58$, $p = 0.013$) (**Fig.2A**) and with cholinesterase plasma levels ($\beta = -0.717$, $p = 0.0018$)(**Fig.2B**), and directly correlate with the pre-operative MELD score ($\beta = 0.425$, $p = 0.043$)**(Fig. 2C)**. Also HMGB-1 baseline plasma levels directly correlated with pre-operative MELD score ($\beta = 0.448$, $p < 0.05$) **(Fig. 2D)**.

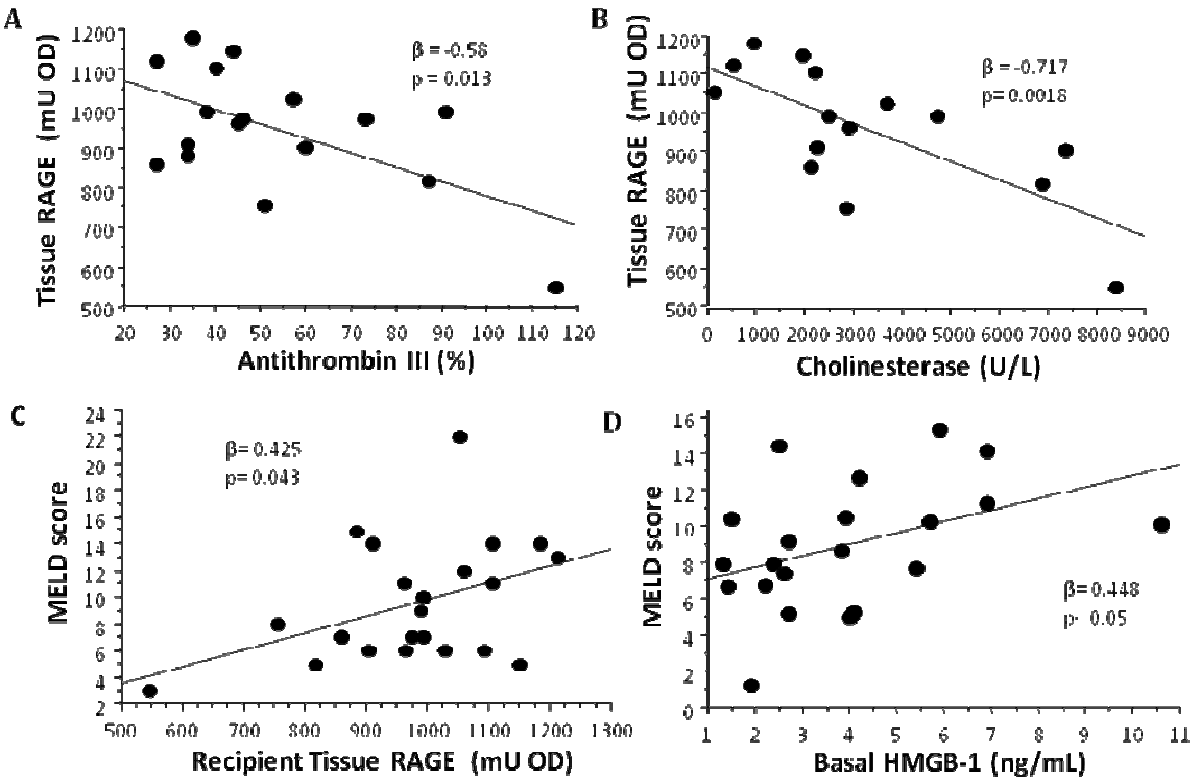

Fig. 2. Hepatic RAGE mRNA expression inversely correlated with antithrombin III **(A)** and cholinesterase plasma levels **(B)**. The preoperative MELD score directly correlated with hepatic RAGE mRNA expression **(C)** and baseline HMGB-1 plasma levels **(D)**.

The pre-operative CML plasma levels were higher in LT recipients than donors (p=0.02) **(Fig 3A)**. After graft reperfusion they significantly decreased (p<0.0001) and quickly returned to basal values one day after LT **(Fig. 3A)**.

Baseline HMGB-1 levels did not differ between recipients and donors; however the values in LT recipients were significantly higher than healthy subjects (3.8 ± 2.3 ng/mL *versus* 0.45 ± 0.3 ng/mL, p<0.0001). The levels of HMGB-1 increased after graft reperfusion (39.9 ±18 ng/mL, p<0.0001) and returned readily to baseline values one day after LT (3.6 ± 2.9 ng/mL) **(Fig 3B)**.

Pre-operative plasma levels of sRAGE did not differ between recipients and donors **(Fig 3C)**. Plasma sRAGE levels did not change soon after LT but decreased dramatically on day 7 ($p < 0.0001$) **(Fig 3C)**.

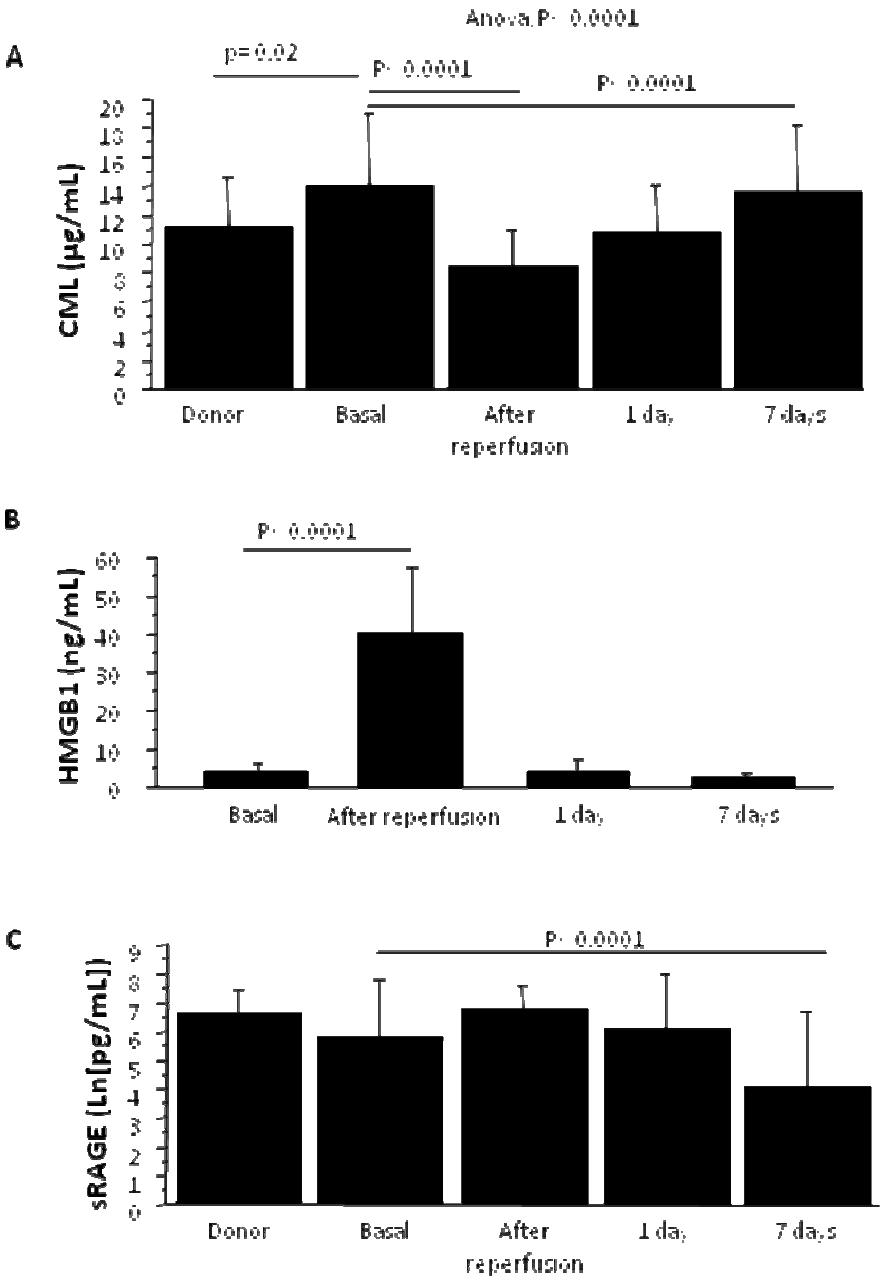

Fig. 3 Comparison between donors and recipients prior to LT in plasma levels of sRAGE (**A**), CML (**B**) and HMGB1(**C**) and kinetics of the same parameters in LT recipients at follow-up.

Using univariate linear regression analysis, the MELD score on day 7 correlated inversely with graft esRAGE mRNA expression (β = -0.48, p = 0.03) **(Table 2)**. Instead, the MELD score on day 7 tended to correlate directly with the peak values of HMGB-1 after reperfusion (β = 0.42, p = 0.07), with recipient age (β = 0.38, p = 0.07) and recipient gender (β = 0.49, p = 0.015) **(Table 2)**. After adjustment for gender, donor age, recipient age, multivariate linear regression analysis showed that only graft esRAGE mRNA expression remained significantly associated with MELD score on day 7 (β = -0.788, p = 0.0005) **(Table 2)**.

Table 2. Simple and multiple regression analysis with MELD score on day 7 as dependent variable

	β	**p value**
	Simple regression analysis	
Age of recipients	0.38	0.07
Age of donors	0.22	0.3
Male of recipients	0.49	0.015
Graft esRAGE mRNA expression	-0.48	0.03
HMGB-1 after reperfusion	0.42	0.07
	Multiple regression analysis	
Graft esRAGE mRNA expression	**-0.788**	**0.0005**
HMGB-1 after reperfusion	0.461	0.013
Age of recipients	0.494	0.02
Male of recipients	0.492	0.02

DISCUSSION

The present study provides the evidence for the **involvement of RAGE and RAGE-ligands in liver transplantation and graft dysfunction**. RAGE tissue levels were higher in LT recipients than donors. In a recent study highest level of hepatic RAGE expression have been found in patients with HCC [72] and since our population was composed mainly by patients with HCC (64%), highest levels of tissue RAGE in recipients could be due to most HCC presence [72]. In our patients, the RAGE tissue levels inversely correlated with antithrombin III and cholinesterase, two plasmatic markers of liver dysfunction that are decreased in patients with liver impairment and whose decrease reflects disease severity [83, 84]. This correlation indicates that **high levels of tissue RAGE can be deleterious for liver survival**. This concept is confirmed by another significant result, i.e. the direct correlation between the MELD score prior to LT, a robust marker of disease severity and mortality, and the tissue RAGE.

Among ligands of RAGE, the most important for the liver impairment is the HMGB-1, a protein present in the nucleus of almost all eukaryotic cells which can be released by cells undergoing necrosis or in response to hypoxia [49, 59]. It has been demonstrated that, during human liver transplantation, circulating levels of HMGB-1 were undetectable before graft reperfusion, increased after reperfusion, and correlated with graft steatosis [77]. In our study, **we confirm the changes of HMGB-1 plasma levels** during peri-operative period, suggesting that HMGB-1 was released by the transplanted liver, maybe following to ischemia/reperfusion (I/R) injury of the organ. The higher values of HMGB-1 in LT recipient than in normal subjects and the positive relationship between HMGB-1 baseline and pre-operative MELD score, underline the importance of

this marker also in chronic liver disease condition [85], so that HMGB-1 could become a prognostic marker of liver dysfunction.

The CML plasma levels were higher in LT recipients than in donors, maybe due to patient liver impairment that leads to a reduction in its physiological function of AGEs clearance. Further, in LT recipients after an immediate significant reduction, plasma CML levels returned to baseline values on day 7 by surgery, underlining the **inability of the transplanted liver to restart its detoxification function**.

There were no significant differences between LT recipients and donors in sRAGE plasma levels. However **circulating sRAGE** did not change significantly soon after LT while **decreased dramatically** on day 7 after LT ($p = 0.0001$) and remained constantly low during follow-up. We do not know the raison of these changes but can assume that they can be due or by an enhanced clearance or by a decreased expression/release, possibly affected by immuno-suppression therapy. Since serum creatinine before and 7 days post-LT does not change, the second hypothesis is more probable and needs to be further investigated. On the other hand, in a circumstance very similar to ours such as kidney transplant, this protective factor was heavily declined and was associated with 2-3 times higher risk for mortality in renal transplant recipients [86].

The most interesting finding, in our opinion, is the inverse association between the expression of esRAGE in donor biopsies with the MELD score on day 7after surgery. Although this relationship does not imply causality, it suggests a possible influence of esRAGE graft expression on graft dysfunction and survival monitored by the post-operative MELD score. Therefore, considering these results as a whole, i.e., the association between graft esRAGE and MELD score on day 7, the rapid increase of HMGB-1 at reperfusion and the CML accumulation in the bloodstream followed by a dramatic decline of the protective sRAGE in early post-operative period, we believe that

this may result in deleterious consequences on graft survival and outcomes of patients. It is so conceivable assume that circulating sRAGE and ligands, tissue esRAGE/RAGE expression could be combined with each other in **an risk scoring algorithm** for graft dysfunction prediction after liver transplantation.

Then RAGE-ligand axis represent a promising target for further investigation as a useful biological marker of liver injury prior to LT and perhaps, in patients with early allograft dysfunction after LT. Identification of patients at risk of a complicated course after LT is crucial for adapting post-operative care. The RAGE–ligand pathway may be a worthwhile therapeutic target with a wider therapeutic window aimed at ameliorating graft function and survival.

Our study has several inherent limitations. Clearly, this work is exploratory in nature and a descriptive study, therefore cannot assign causality or mechanism to our findings, nor can we recommend specific clinical interventions or action based on our conclusions. The sample size is the major limiting factor, and a prospective validation set is still needed for more definitive conclusions and to confirm any clinical associations. We also recognize some limitations in missed early clinical time points in the first week after LT.

Despite these limitations, **this study provides evidence for several associations between specific inflammatory mediators, linked to RAGE, and graft dysfunction that can guide future investigation.**

In conclusion, the plasma levels of sRAGE, HMGB-1 and CML and the tissue levels of RAGE and esRAGE in recipient and in liver graft, may be promising targets for further investigation as mediators of liver injury post-transplantation, and serve as potential clinical biomarkers for prediction of early detection of graft dysfunction and the possibility of graft loss.

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