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TESI DI DOTTORATO

Molecular mechanisms of HMGB1, a key mediator of inflammation, and role in diseases such as cancer and diabetes mellitus

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Alle persone che più amo e alla mia
piccola stellina, che mi ha lasciato
un vuoto troppo grande. Mi manchi.

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BACKGROUND

HMGB1 IN HUMAN DISEASE LIKE CANCER AND DIABETES MELLITUS.

The high mobility group box 1 (HMGB1) protein is an abundant non-histone component of chromatin, well known for its two DNA binding domains, HMG box A and HMG box B. The main ability of the HMGB1 protein is to recognize and bind with high affinity to DNA. The HMGB1 protein functions have been correlated to cancer progression, in fact an elevated expression of HMGB1 occurred in certain types of primary tumors, including melanoma and in the majority of cases HMGB1 is associated with invasion and metastasis. The protein was proposed to be directly involved in tumor cell metastasis through its ability to promote cell migration, modulate the adhesive properties of cells and modify components of the extracellular matrix **(Todorova et al. 2012).**

We know for sure that the main signalling pathway is activated through the interaction with a Receptor for Advanced Glycation End products (RAGE).

RAGE is a member of the immunoglobulin superfamily of cell surface molecules and consists of three extracellular immunoglobulin domains V, C1 and C2, a transmembrane helix and a short cytosolic tail **(Marion et al. 1999)**.

RAGE is expressed in a wide range of tissues such as lung, heart, kidney, brain, skeletal muscles and in different types of cells including endothelial cells, macrophages/monocytes, neutrophils, and lymphocytes. With AGEs interaction, RAGE mediate the effects of its other extracellular ligands (extracellular high mobility group box-1 (HMGB1), S100 family of calcium binding proteins among many others). Although a large number of advanced glycation end products have been identified in humans, AGE/RAGE signaling ex-vivo is mostly studied using the AGEs such as AGE-modified albumin.

The discovery that RAGE bound "amphoterin" or HMGB1 suggested for the first time roles for RAGE in cellular migration and

invasiveness, initially in the context of neurite outgrowth **(Ramasamy et al. 2009).**

RAGE interacts with various structurally different ligands, including advanced glycation end products (AGEs), amyloid β peptide (Aβ), transthyretin, amphoterin and members of the S100 protein family (S100 proteins regulate intracellular processes such as cell growth and motility, cell cycle regulation, transcription and differentiation) **(Sárkány et al. 2011).**

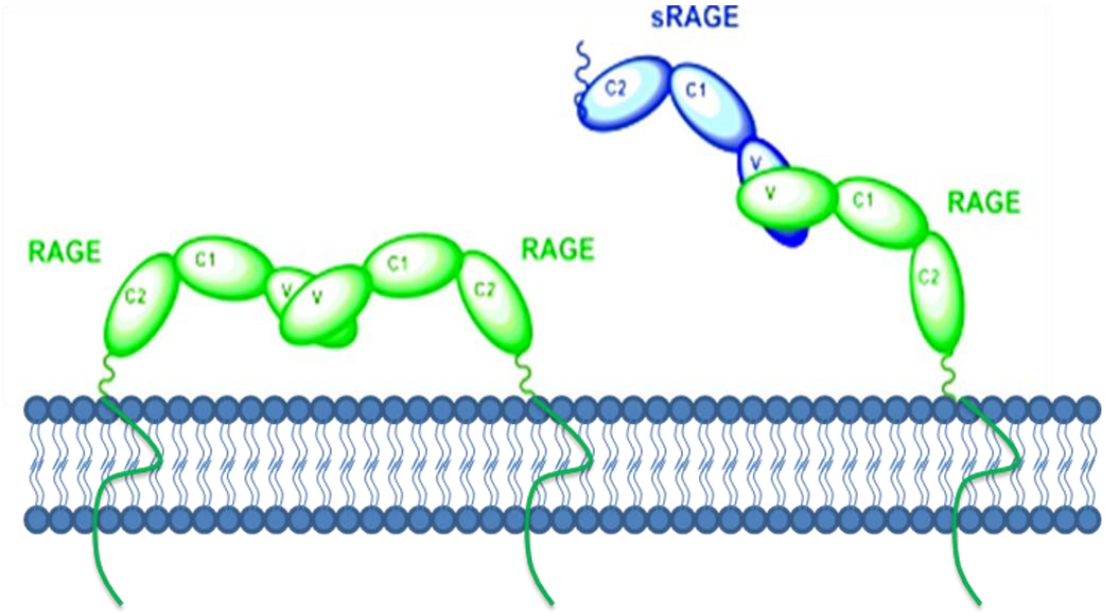

FIGURE 1: Associations of sRAGE (RAGE extracellular region, soluble protein, may contribute to the removal/neutralization of circulating ligands) and RAGE with different aspects of metabolic, vascular and autoimmune disease.

It is speculated, that the soluble forms of RAGE might counteract inflammatory reflexes triggered by RAGE ligands such as AGEs, S100 proteins and HMGB1 **(Humpert et al. 2007).**

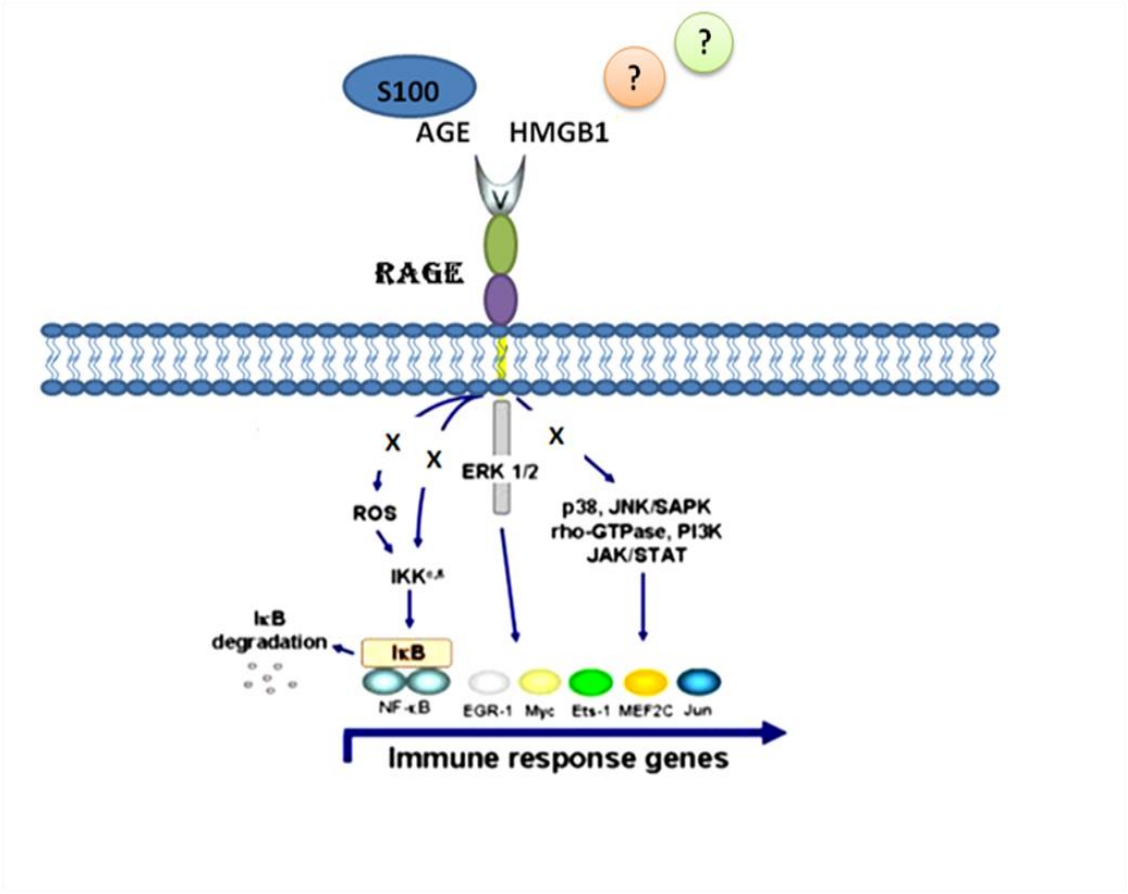

FIGURE 2: Receptor of advanced glycation end products (RAGE)-mediated signal transduction. AGE, advanced glycation end product; HMGB1, high-mobility group box protein 1; IκB, inhibitor of kappa B; IKK, inhibitor of kappa B kinase; JAK, Janus kinase; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species.

HMGB1 is able to activate other cells involved in immune response or inflammatory reactions, thus acting as a cytokine itself. The protein can also be passively released by damaged or necrotic cells, leading to inflammation. A number of post-translational modifications including acetylation, phosphorylation, methylation and poly(ADP)-ribosylation have been suggested to direct HMGB1 to the secretory pathway **(Todorova et al. 2012).**

All these considerations indicate that HMGB1 is a very important mediator of inflammatory response, with several mechanisms of action not yet completely elucidated.

DIABETES MELLITUS

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, skin and blood vessels. Several pathogenic processes are involved in the

development of diabetes e.g. autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency, or other abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues.

Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycaemia.

DM is characterized by the onset of acute and long-term complications, the latter including retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers and amputations; autonomic neuropathy causing gastrointestinal, genitourinary and cardiovascular symptoms and sexual dysfunctions. Moreover patients with diabetes have an increased incidence of atherosclerosis and cardiovascular diseases. **(American Diabetes Association 2012).**

There are two types of diabetes:

- Type 1 diabetes (T1D), is an autoimmune disorder characterized by the specific destruction of the insulin secreting beta cells of the pancreatic islets by a certain population of autoreactive immune cells. About 10% of the total number of DM patients suffer for this type of diabetes which is also called juvenile diabetes, for the age of onset, and insulin-dependent, due to the pathogenetic mechanisms. The therapy is mainly related to the insulin replacement. Insulin is a hormone that is needed to convert sugar, starches and other food into energy needed for daily life. With the help of insulin therapy and other treatments, even young children with type 1 diabetes can learn to manage their condition and live long, healthy, happy lives **(http://www.diabetes.org/diabetes-basics/type-1/)**.
- Type 2 diabetes (T2D), the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. A degree of hyperglycaemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms, may be

present for a long period of time before diabetes is detected. During this asymptomatic period, it is possible to demonstrate an abnormality in carbohydrate metabolism by measurement of plasma glucose in the fasting state or after a challenge with an oral glucose load.

The degree of hyperglycaemia may change over time, depending on the extent of the underlying disease process. The same disease process can cause impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) without fulfilling the criteria for the diagnosis of diabetes. In some individuals with DM, adequate glycemic control can be achieved with diet, weight reduction, exercise, and/or oral glucose-lowering agents. These individuals therefore do not require insulin. Other individuals who have some residual insulin secretion but require exogenous insulin for adequate glycemic control can survive without it. Individuals with extensive β-cell destruction and therefore no residual insulin secretion require insulin for survival.

Thus, the degree of hyperglycaemia reflects the severity of the underlying metabolic process and its treatment more than the nature of the process itself.

PATHOGENETIC MECHANISMS OF DIABETIC COMPLICATIONS

During diabetes, hyperglycaemia affects the cells of all tissues but the detrimental effects of this metabolic disorder can be selectively observed in the insulin-independent tissues in which the glucose uptake into the cells does not require the action of insulin leading to a high intracellular concentration of the sugar.

In the past decades, several studies demonstrated that the pathogenetic cascade of diabetes-induced complications is related to the metabolic consequences of excess glucose disposal, either through repeated acute changes in cellular glucose metabolism, or through the long-term accumulation of altered products.

In detail, acute hyperglycaemia is characterized by an increase of biomarkers of oxidative stress and cytokine secretion, which are

concomitant with a decreased activity of antioxidant systems induced by glucose intake **(Vlassara et al. 2002).**

Chronic exposure to high glucose is essentially linked to the formation of glycated biomolecules and the progressive accumulation of Advanced Glycation End-products (AGEs) through the augmentation of the entity of the chemical (non enzymatic) reaction of reducing sugars with amino groups. The Maillard reaction is also known as glycation, **(FIGURE 3)** which leads to the formation of early, intermediate (Schiff base, Amadori) and advanced glycation products **(Ramasamy et al. 2011; Pricci et al. 2000).**

Further, the interaction between AGEs and receptor for AGEs (RAGE) also cause inflammation and oxidative stress **(Nagai et al. 2012).**

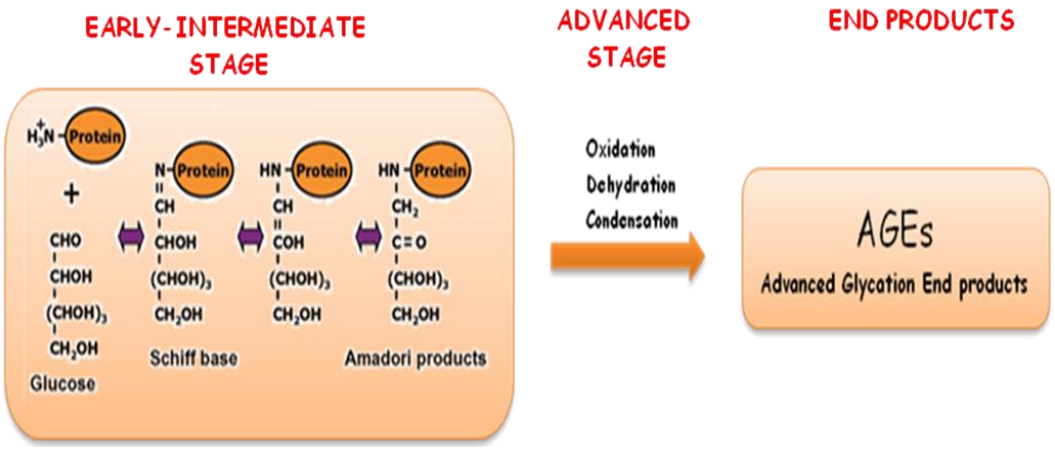

FIGURE 3: The Maillard reaction occurs in three main steps: Initial step is formation of N glycoside the immonium ion is formed and then isomerize, this reaction is called Amadori rearrangement and forms a compound called ketosamine.

Finally, this pathway has been demonstrated to lead to oxidative stress and pro-inflammatory signalling implicated in endothelium dysfunction, arterial stiffening, and microvascular complications **(Vlassara et al. 2002; Stitt et al. 2003; Scivittaro et al. 2000).**

Recent studies have demonstrated that the non-enzymatic glycation is not exclusively linked to the high availability of glucose, but numerous intermediate metabolites, such as a-ossoaldehydes (for example the methylglyoxal), generated during glycolysis, the polyol

pathway, or the autoxidation of carbohydrates (glyoxal), may participate in the non-enzymatic glycation **(FIGURE 4)**.

The α-ossoaldehydes, for instance, create AGE surprisingly quickly, unlike the Maillard reaction, which is significantly slower **(Leto et al. 2001)**.

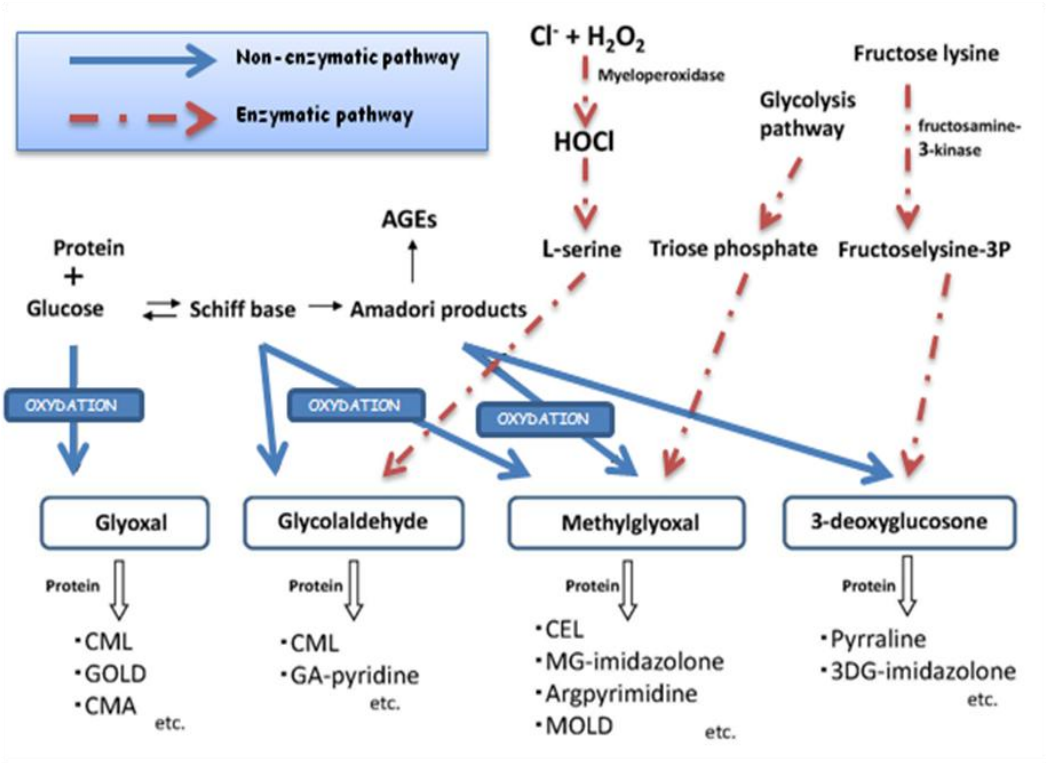

FIGURE 4: Pathway of intermediated aldehyde generation.

The predominant route in AGE-products formation may depend on many variables, including the type of tissue, the species, the age of

the organism and diet, as well as those variables that induce oxidative stress.

In diabetic conditions a metabolic anomalies puts the oxidative stress as the central mechanism (acute and in chronic exposure to high glucose) leading to the cross-connection of all these metabolic pathways **(Nishikawa et al. 2000; Brownlee et al. 2001).**

In fact, High Glucose-dependent metabolic alterations have been demonstrated to lead to an imbalance in several cytokines and signalling molecules, depicting a chronic low-grade inflammation pattern, observed in diabetes-affected tissues. In fact the damaging effects of hyperglycaemia affect mainly certain cell types that (a) are unable to maintain their intracellular glucose concentration in hyperglycaemic conditions, and particularly cell types involved in diabetic complications (e.g. endothelials and neurons) and (b) generate high Reactive Oxygen Species (ROS) levels and induce a huge oxidative stress implicated in glycation, AGEs formation, and AGE=RAGE interactions **(Negre-Salvayre et al. 2009).**

In summary, the metabolic alterations induced by high glucose are time-dependent but also strictly interrelated, thus making difficult to separate a single pathway responsible to the pathogenesis of diabetic complications.

Regarding the mechanisms of eye diabetic complications, many studies examined the mechanisms underlying vasculopathy and found that all the metabolic alterations listed below are present and lead to vessel alterations and subsequent neo-angiogenesis, justifying the different progressive phases of DR (Diabetic retinopathy). On the contrary, little is known about the metabolic aspects of excess glucose disposal in neuroglial compartment. Another very interesting aspect is the observation that hyperglycaemic diabetic mice show an impaired angiogenic response **(Facchiano et al. 2002 and 2006)** likely due to glycation of basic FGF, one of the most potent angiogenic factors, or of other key factors. These studies suggested that the angiogenesis dys-regulation (a key event both in cancer and in DM complications) may be directly related to hyperglycaemia and AGE-products accumulation.

THE ROLE OF HMGB1 IN CANCER

DM has been recognized as a key factor contributing to the development of solid organ malignancies including liver, pancreas, colorectal, breast, endometrial, uterine, and bladder. The two cancers showing the strongest association to T2D are those involving liver and pancreas. Many studies demonstrated increased cancer risk and mortality among individuals with T2D, especially those with poorly controlled T2D based on fasting serum glucose levels. A recent meta-analysis of 23 articles indicated a 41% increase in cancer mortality related to endometrial, breast, and colorectal cancer in patients with pre-existing diabetes as compared to normoglycemic individuals **(Grace et al. 2011).**

These studies suggest that the study of pro-inflammatory signals related to impaired glucose controls and AGE-products accumulation, likely involving a key role of the AGE-RAGE-HMGB1 axis, is a very interesting and fascinating field of study. Therefore it represents one of the main focus of this PhD. Thesis.

HMGB1 STRUCTURE, MODIFICATION AND EXPRESSION

High-mobility group box protein 1 (HMGB1) belongs to HMG family that are typically 25 to 30 kDa molecular weight proteins. The superfamily consist of 3 families: HMGA, HMGB and HMGN.

The HMGB family has three domains:

- HMG boxes A and boxes B (~75 amino acids);
- C-terminal domain is highly negatively charged (glutamate and aspartate residues) **(Agresti et al. 2003) (FIGURE 5)**.

HMGB1 is a highly conserved nuclear protein that is a prototype for a unique class of pro-inflammatory mediators called

ALARMINS. As a group, alarmins display distinct intracellular and extracellular activities, with potent stimulation of the innate immune system as their cardinal feature.

Amino acid composition of HMGB1:

```
1           11          21          31          41
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKKEEEED EEDEEDEEEE
EDEEDEDEEE DDDDE
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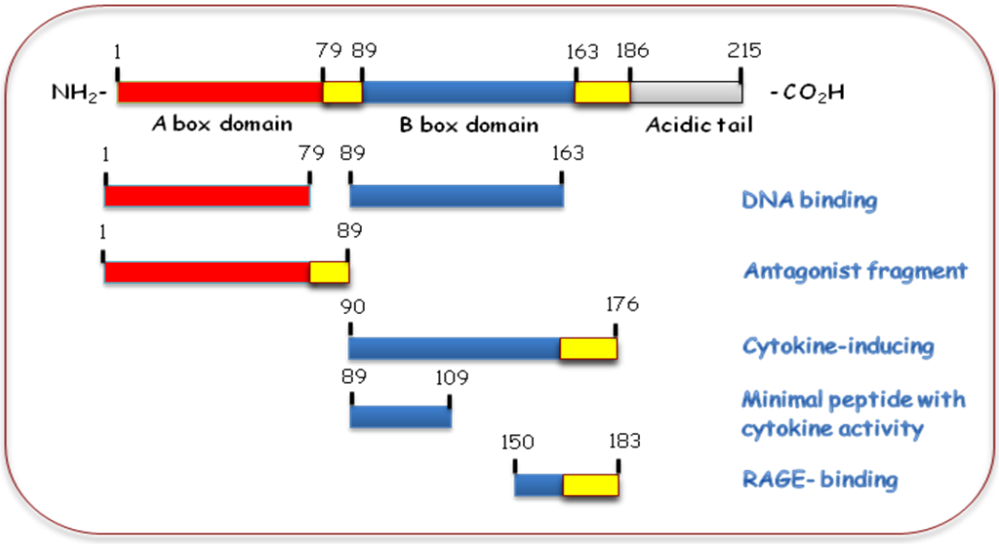


FIGURE 5: Structures of HMGB1 protein domains.

While the intracellular functions of alarmins vary, in their extracellular form, they function as pro-inflammatory mediators to alert the immune system to tissue damage and to trigger an immediate response. A key feature of the biology of alarmins is therefore their translocation from inside to outside of the cell.

Human HMGB1, a 215-amino acid protein, contains three major functional domains: the A box, the B box, and the C-terminal acidic

tail (C tail). Structure-function analyses reveal that the pro-inflammatory cytokine inducing capacity of HMGB1 localizes to the B box, with the most significant cytokine functionality mapping in the first 20 amino acid residues of this domain (A box domain). HMG boxes A and B are each ~75 amino acids in length, the C-terminal domain is highly negatively charged, consisting of a continuous stretch of glutamate and aspartate residues **(Agresti et al. 2003)**.

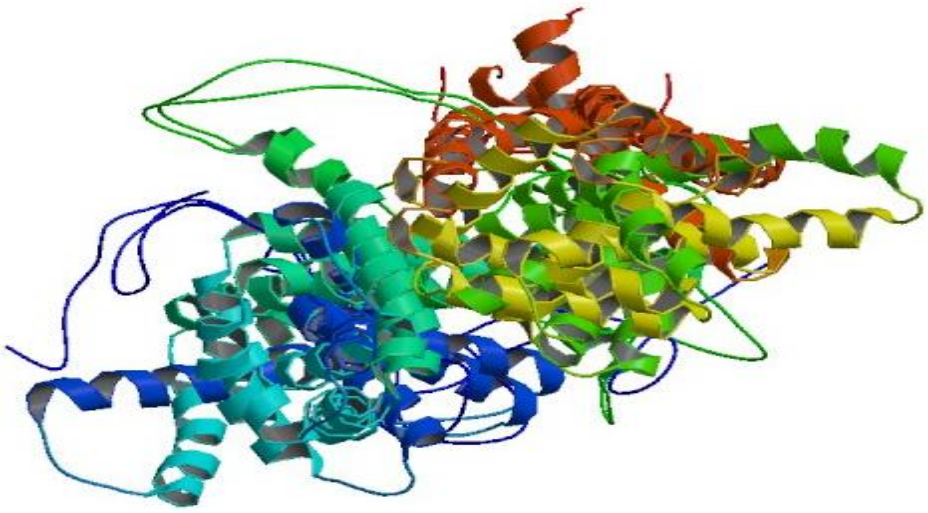


FIGURE 6: Ribbon model of human HMGB1.

NUCLEAR FUNCTIONS OF HMGB1

The HMGB protein contains HMG boxes that bind DNA.

HMGB1 binds rather weakly to the common B-form variety to DNA and hasn't sequence specificity. Instead, it binds with high affinity to unusual DNA structure like 4-way junctions and DNA bulges. This suggests that HMGB1 might actually function by distorting linear DNA into a bent conformation and that it recognized molecules that resemble transition state between reagents and products.

The intranuclear concentration of HMGB1 is in micromolar range, when HMGB1 concentration will increase the fraction of interacting partner molecules. HMGB1 play a role organizing enhanceosomes it can modulate the other key factor in transcriptional regulation: chromatin remodeling. HMGB1 and other chromatin protein like histone H1, can interact with nucleasomes at the entry end exit points of DNA. The HMGB1-DNA binding is dynamic and reversible in a chaperone mode **(Agresti et al., 2003).**

RECEPTORS ASSOCIATED WITH HMGB1 SIGNALING PATHWAYS

There are several important receptors have been characterized to be implicated in HMGB1 signaling, including the receptor for advanced glycation end products (RAGE) and some members of the Toll-like family of receptors. The first identified cellular receptor for this nuclear protein was the receptor for advanced glycation end products (RAGE), which mediates the interactions between advanced glycation end product (AGE)–modified proteins and the endothelium and other cell types **(Hori et al 1995)**.

RAGE is a transmembrane protein expressed at low levels in normal tissues, but it is up-regulated at sites where its ligands accumulate **(Chavakis et al. 2004)**.

Binding of RAGE by HMGB1 can activate both CDC42/Rac pathway and MAPKs-NFκB pathway **(Yang et al. 2005)**.

These two HMGB1 signaling pathways then through RAGE promotes chemotaxis, production of cytokines relevant to NF-κB activation,

activation of endothelial cells, maturation and migration of immune cells **(FIGURE 2)**.

There are additional receptor(s) relevant to HMGB1 signaling. Consistent with this assumption, follow up studies further characterized that toll-like receptors 2 (TLR2) and 4 (TLR4) are involved in HMGB1 signaling **(Shu Zhang et al 2010)** likely through Rac1/PI3K/CDC42 pathway and MyD88 dependent NF-κB activation pathways.

ADVANCED GLYCATION END PRODUCTS (AGE)

The **Advanced Glycation End Products (AGE)** are the result of a non-enzymatic reaction between reducing sugars and cellular components (Proteins, nucleic acid, lipids and other molecules) **(Munch et al. 1997)**.

For example, glucose (or other reducing agents) and a free amino group form reversible intermediates of a Schiff base and an

Amadori product before a series of reactions that irreversibly generate an AGE **(Huebschmann et al. 2006).**

But there are more pathways for AGEs formation:

- the "carbonyl stress" pathway, where oxidation of sugars and/or lipids create dicarbonyl intermediate compounds that use highly reactive carbonyl groups to bind amino acids and form AGEs.
- Monocytes, macrophages, and dendritic cells secrete the nuclear protein amphoterin (similar to HMGB1) to amplify inflammation reaction **(Huebschmann et al. 2006);**
- neutrophils, monocytes, and macrophages, upon inflammatory stimulation (reaction non–glucose-dependent) produce myeloperoxidase and NADPH oxidase enzymes that induce AGE formation by oxidizing amino acids **(Huebschmann et al. 2006).**
- Receptors for AGE (RAGE) when bound to AGEs, associated with reactive oxygen species (ROS) generation promote more AGEs via the NADPH oxidase pathway **(Ramasamy R et al. 2011; Wautier MP et al. 2001).**

- The aldose reductase–mediated polyol pathway may directly form AGEs via 3-deoxyglucosone AGE intermediates. This reaction causes depletion of NADPH and glutathione, and the resultant oxidative stress indirectly increases formation of AGEs **(Huebschmann et al. 2006).**

AGEs can cause extensive cross-linking of proteins its oxidation, leading to their deposition, as reported in diabetes and Alzheimer's diseases **(Smith et al. 1995; Gella et al. 2009).**

All together these reports indicates that HMGB1's role in the mechanisms of inflammation, apoptosis, angiogenesis, DM and cancer, are very important although still poorly known.

Therefore it is very important to investigate such aspects to better understand these pathogenetic mechanisms and possibly identify the molecular pathways involved, with the aim to develop novel therapeutic strategies for these serious diseases.

PhD PROJECT

AIMS

This study aims to identify proteins that interact with HMGB1, an alarmine involved in the metabolic pathways of inflammation, as previously described. The research is based, on the achievement of the following 3 aims:

1) the designation of a proper experimental model;

2) the development of methods for obtaining a right stimulus (AGE accumulation) to study the HMGB1 effects on our models;

3) the study of the HMGB1-interactome through the use of immunoprecipitation methods to be specifically developed coupled to SDS-PAGE techniques and mass spectrometry to identify novel proteins involved in the interaction with HMGB1.

MATERIALS AND METHODS

IN VITRO GLYCATION

The Bovine Serum Albumin (BSA, Sigma Aldrich. St. Louis, Mo) was prepared as previously reported **(Pugliese et al. 2008; De Oliveira Silva et al. 2008).**

Briefly, 50 mg/ml BSA solution in 0.2 M phosphate buffer, pH 7.8, containing antibiotics (10,000 U.I penicillin and 10,000 µg/ml streptomycin) and protease inhibitors (1 mM EDTA) was incubated with (glycated) or without (non-glycated) 500 mM D-glucose for 8 weeks at 37 °C under sterile conditions. Glycated-BSA (AGE-BSA) and non glycated BSA (BSA) were dialyzed against water for 24 hours using dialysis membranes (PBI International, MWCO 12,000–14,000, Milano, Italia), the solution was passed through 22 µm filters, assessed for endotoxin content by the Limulus amoebocyte lysate assay (SIGMA) and, finally, for AGE levels measurements by fluorescence and for protein content by Coomassie Protein Assay Kit (Pierce, 3747 N. Meridian Road P.O. Box 117Rockford, IL 61105).

CELL COLTURE AND TREATMENTS

IN VIVO STUDIES

The experimental activity on animal models was carried out in accordance with the "Principles of Laboratory Animal Care" **(NIH Publication nos. 85–23, revised 1996)** and national laws.

In vivo studies were performed using male Sprague-Dawley rats (Harlan Laboratories, Indianapolis) rendered diabetic with streptozotocine (STZ) Sigma) a molecule able to induce massive destruction of pancreatic β cells.

The STZ, in buffer solution of 0.1 M sodium-citrate (pH 4.5) freshly prepared, was used in an amount equal to 60 mg / Kg of weight in animals fasted for about 4 hours and age-matched control rats, studied at different times of disease (1, 3, 6, 12 weeks).

At the end of the experimental procedures, eyes were enucleated from each rats and the retina of one eye was isolated for the protein studies whereas the other eye was frozen for the tissue sectioning. Blood was sampled for the analysis of biochemical

parameters of diabetes (fasting glucose, glycated hemoglobin, AGEs measurement).

RETINA FROM EYEBULBS

The retinal tissue and eyebulb were fixed in 4% paraformaldehyde in PBS, 0.12 M in sucrose for 30 min or overnight. After fixation, the samples were rinsed for three times in PBS, with 5% sucrose and 0.15 mM $CaCl_2$ and left overnight in sucrose buffer (PBS with 30% sucrose and 0.15 mM $CaCl_2$). Samples were then embedded in Tissue Freezing Medium (Jung, Germany), frozen at –30 °C in isopentane and stored at –80 °C. Sections were cut at a Reichert–Jung Frigocut cryostat, stained with hematoxylin and observed at Nikon Optiphot microscope equipped with a video confocal (ViCo) System.

RETINAL TISSUE CULTURES AND LYSIS

Retinal explants were obtained from 7–8 week-old male Sprague-Dawley rats. Retinas were dissected according to the protocol described by Pinzon-Duarte **(Pinzon-Duarte et al 2000)**.

Briefly, the eyes were enucleated from rats under deep anaesthesia and transferred to a petri dish (Corning Incorporated NY, USA) containing ice-cold sterile phosphate-buffered saline (PBS) and incised just dorsally to the ora serrata. Cornea and lens were removed and the whole retina was carefully dissected from the sclera, placed flat onto a PET microporous insert (Transwell, Corning Incorporated NY, USA), to allow the tissue being in contact with the culture medium at both the apical and basal sides, and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco srl, Paisley, Scotland, UK) containing 10% fetal calf serum (FCS, Life Technologies, Milan, Italy) and antibiotics, in a tissue culture incubator at 37°C, 5% CO_2.

Retinal cells or tissue were preparated as describe in Villa M. PhD Thesis.

HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVEC) were from (American Type culture Collection, ATCC, Manassas, VA) and were cultured and passaged as described **(Facchiano et al. 2001)** in endothelial growth medium (EGM)-2 containing growth factors (Hyclone, South Logan, UT), cells were used between passage 3 and 6. Cells ($5x10^5$) were plated in 10 cm Petri dishes or in 6 well plastic plates (Corning Incorporated) in the presence of 10% fetal calf serum (FCS) containing medium, and after 24 hours growth cells were thereafter grown for additional 24-48-72 hours in the absence of FCS, but in the presence of AGE or control BSA. At the end of this time, cell extracts were produced as described **(Facchiano et al. 2012)**.

The HUVEC were treated a time zero (t0) with 200 μg/ml of Bovine Serum Albumine (BSA) with advanced glycation end products (BSA-AGE) (preparated by incubation with D-Glucose 500 mM for 8 weeks as above described), BSA 200 μg/ml like a control of BSA-AGE, Normal Glucose (NG) 5.5 mM corresponding to

normoglycaemia, High Glucose (HG) 30mM corresponding to high levels of glycemia, Mannitol 30mM (in distilled water, Sigma).

A375 MELANOMA CELLS

The A375 melanoma cells (American Type culture Collection, ATCC, Manassas, VA) were propagated under standard culture conditions. Cultures were found to be free from Mycoplasma species using the Hoechst staining procedure. The A375 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, South Logan, UT) at pH 7.4, containing FBS (fetal bovine serum) (Sigma-Aldrich, St. Louis, MO); at 10%, L-glutamine 2 mM and penicillin / streptomycin (100 U / ml) (Invitrogen, Carlsbad, CA) and when required in conditions of absence of serum.

These cells were propagated according to standard culture conditions **(Facchiano et al. 2012)** using 75 cm^2 flasks.

The invasive potential was assessed by in vitro invasion assay as described **(Albini et al. 1987).**

The metastatic activity, both spontaneous and experimental, was found to be stable over the time of the experimental procedures.

Cell viability was tested by the Trypan Blue (0.25 %) exclusion test at different times of growth. At the end of the treatment, cells were photographed and harvested, then cell extracts were produced as described **(Facchiano et al. 2012)**.

CELL LYSIS

The process of cell lysis takes place totally in ice using 500 μL of lysis buffer and collecting the cells by the aid of a "scraper".

The sample is maintained for 20 minutes on ice and then subjected to centrifugation at 4 ° C for 10 minutes at 13,200 rpm in an Eppendorf centrifuge 5415R Centrifuge in order to remove the excess of nucleic acids. Then remove the supernatant with a pipette tip made of polypropylene from 200 mL and these samples are then stored at -80 °C.

PROTEIN GEL ELECTROPHORESIS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyze proteins based on differences in their molecular size, according to a published protocol **(Facchiano et al. 2010).**

Briefly, our analysis on SDS-PAGE analysis was performed using 2.4% -18% acrylamide-bisacrylamide gradient. The gels were obtained by the polymerization of acrylamide and of 'N, N'-methylene-bisacrilamide (Bis-acrylamide), which acts by crosslinking of linear chains. The polymerization is due to the presence of free radicals, provided by the persulphate ammonium (APS), stabilized by 'N, N, N', N'-tetra-methylethylene-diamine (TEMED).

Each lane was loaded with 25 μg of total proteins of each sample (protein concentration was performed with Bradford methods), previously denatured by boiling for 5 min at 100 °C with the addition of sample buffer (44 mM Tris-HCl pH 6.8, 2% SDS (w/v),

10% Glycerol (v/v), 5% 2-β-mercaptoethanol (v/v) and 0.0125% Bromophenol Blue (w/v)), it was run into running buffer (25 mM Tris, 250 mM glicina, 0,1% SDS) at 200V constant voltage.

The gel was then fixed with fixing solution (60% H_2O, 30% Methanol, 10% Acetic Acid) and then stained with Coomassie (Brilliant Blue, acetic acid, methanol, water) **(Facchiano et al 2012).**

IMMUMOPRECIPITATION

Immunoprecipitation (IP) is a method that uses the antigen-antibody interaction principle to isolate a protein that reacts specifically with an antibody from mixture of proteins.

The IP protocol was performed as follows:

First step (Pre-clearing):

- the lysate (800 μg of total proteins) was incubated with beads (protein A/G GE Healthcare Milwaukee USA previously washed with PBS) for 3 hours at room temperature under rotation: This can help to reduce the background.

The second step: *beads-antibody reaction*:

- The antibody (anti-HMGB1 Abcam 79823 Cambridge, UK) was incubated with beads over-night at +4°C in rotation according to the manufacturer's instructions.

The third step: *pre-cleared and "activated beads" incubation*:

- The pre-cleared sample was incubated with conjugated beads (*activated beads*) for 3 hours at room temperature under rotating conditions.

  Then the tube was centrifugated, the supernatant was collected and at the beads (the antibody binding a proteins) added with sample buffer then denatured at 100 °C as above reported.

After a centrifugation the supernatant was collected (IP) and ready to be loaded in the gel.

The immunoprecipitation was performed by using beads (GE Healthcare) pre-incubated for 12 hours at 4 °C in a rotating wheel, with anti-human HMGB1 antibody (Abcam) at the following ratio 40 microliters of Beads : 2 micrograms of antibody . In this way a conjugated Bead-IgG complex was abtained. Then the cell lysates

were incubated with beads-antibody complex for 12 hours at 4°C. Thereafter 3 washes were performed with PBS and the finally release of the antibody-antigen complex was achieved by adding 30 µl sample buffer 4X (β-mercaptanol, SDS 20% glycerol, 1.5M Tris pH 6.8, Bromophenol blue) and boiling for 10 min at 100 °C. The immunoprecipotated were then analyzed by SDS-PAGE Gradient gel (4-15% Biorad, Hercules, CA).

IN GEL DIGESTION

All bands were cut in a piece of the 1 mm^3 and puts in a 1.5 ml eppendorf tube. The bands were washed, 2 times with 50 mM ammonium bicarbonate NH4-HCO3. The next wash was made with ammonium bicarbonate/Acetonitrile CH3CN (1:1). The proteins were then subjected to reduction by 10 mM DTT, and alkylation (alkylating agents that binds the thiol group of cysteines, thereby avoiding that can form disulfide bonds) by 55 mM IAM

(Iodacedammide) in 50 mM ammonium bicarbonate for 30 minutes at room temperature in the dark.

The bands were shrunk in acetonitrile, dried under vacuum, reidrates with trypsin for 45 minutes in the ice and finally digested overnight with (Roche Milan, Italy) bovine pancreatic trypsin, a protease that is part of the family of serine-protease, which cuts specifically at the level of specific residues of arginine (Arg) or lysine (Lys). In particular, the trypsin cuts the peptide bond on the carboxyl side of Arg and Lys residues, so all the protein fragments of a given protein will have at the C-terminal either arginine or lysine, with the exception of the C-terminus of the polypeptide chain original.

The tryptic peptide mixtures obtained were analyzed by nanoLC-MS/MS and Bioworks software (version 3.3, Thermo Electron Fremont, CA).

WESTERN BLOT ANALYSIS

The proteins separated on gel were transferred by electrophoresis at 300 milliAmpere for 30 minutes on a nitrocellulose sheet (0.2 μm, Schleicherm & Schuell Whatman Gmbh, Dassel, Germany) in transfer buffer (Tris-Glicine).

To lock the hydrophilic sites that bind nonspecifically, the nitrocellulose was saturated at room temperature for 3 h with buffer Tween / Tris-buffered Saline (TPBS) containing milk powder 5%. After washing, the nitrocellulose was incubated with the specific primary antibody in a 1.25% solution low fatty acid powder milk +TPBS by stirring.

The primary antibody, polyclonal rabbit anti-HMGB1 (Abcam) was used at a concentration of 1:500 and was incubated for 12 h at +4 ° C. 3 washes were performed of 15 minutes each in TPBS. The nitrocellulose was then incubated with an anti-mouse secondary antibody labeled with peroxidase for 1 h at room temperature.

In order to highlight a reference signal that indicates the amount of protein actually present on the nitrocellulose filter was used the

mouse monoclonal antibody anti-actin (Oncogen); secondary antibody specific incubated for 1 h at room temperature.

The antigen-antibody reaction was detected by a chemiluminescence reaction (Enhanced ChemioLuminescence, PIERCE).

MASS SPECTROMETRY ANALYSIS

Peptide mixtures were analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) using an HPLC Ultimate 3000 (DIONEX, Sunnyvale, CA U.S.A) connected on line with a linear Ion Trap (LTQ, Thermo Electron, San Jose, CA). Peptides have been desalted in a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) and then separated in a reverse phase column, a 10 cm long fused silica capillary (Silica Tips FS 360-75-8, New Objective, Woburn, MA, USA), slurry-packed in-house with 5 µm, 200 Å pore size C18 resin (Michrom Bio Resources, CA). Peptides were eluted using a linear gradient from 96% A (H2O with 5% acetonitrile and 0.1% formic acid) to 60%B (ACN with 5% H2O

and 0.1% formic acid) in 40 min, at 300nl/min flow rate. Analyses were performed in positive ion mode and the HV Potential was set up around 1.7-1.8kV. Full MS spectra ranging from m/z 400 to 2000 Da were acquired in the LTQ mass spectrometer operating in a data-dependent mode in which each full MS scan was followed by five MS/MS scans where the five most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. Target ions already fragmented were dynamically excluded for 30 s. Tandem mass spectra were matched against Swissprot Database, and through SEQUEST algorithm **(Yates et al., 1995)** incorporated in Bioworks software using fully tryptic cleavage constraints with the possibility to have one miss cleavage permitted, static carbamidomethylation on cysteine residues and methionine oxidation as variable modification. Data were searched with 1.5 Da and 1 Da tolerance respectively for precursor and fragment ions. A peptide has been considered legitimately identified when it achieved cross correlation scores of 1.8 for [M+H]1+, 2.5 for [M+2H]2+, 3 for [M+3H]3+, and a peptide

probability cut-off for randomized identification of $p<0.001$. Protein and peptide false discovery rate (FDR) has been calculated dividing the number of false hits by the number of positive hits where the false hits are evaluated using a decoy database directly constructed by Bioworks software on the same human database used for the target search and adopting the same scoring criteria.

MULTIPLEX ANALYSIS OF CELL EXTRACTS AND SUPERNATANTS AFTER AGE EXPOSURE

Samples were collected by cellular lysis and immediately frozen at -80 °C. Before starting the analysis, samples of such cell extracts were thawed at room temperature, then diluted with PBS w/o calcium and magnesium (Euroclone, Milan, IT) and carefully re-suspended by 7-8 passages through a 26 gauge needle. The protein concentration was then measured by Bradford's procedure (Bio-Rad protein assay dye reagent concentration, Biorad Laboratories, Hercules, CA) and a cuvette spectrophotometer (Kontron, Uvikon

860 Instrument Zurich, CH). Samples were then analyzed using the Bio-Plex Pro Human Cytokine 27-plex panel (Biorad) developed to achieve a quantitative measurement of the following analytes: IL-1Ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, TNF-α, IFN-γ, MIP-1α, MIP-1β, eotaxin, MCP-1, G-CSF, GM-CSF, basic FGF, VEGF, IP-10, RANTES, and PDGFbb. Next, 15 µl of the sample were diluted 4-fold with a Bio-plex sample diluent according to the manufacturer's instructions. This procedure was carried out using 50 µl of the diluted sample. After the incubation with antibodies-activated magnetic beads, samples were washed using a Bio-Plex Pro TM Station (Biorad). The quantification was carried out on a Bio-plex Array Reader scanner for Luminex 100 (Luminex, Biorad) by a Bio-Plex Manager Software version 4.1.1.

Results expressed as picograms/ml were analyzed by a Bio-Plex Manager Software version 6. When required, normalization of samples was achieved by correcting for the protein concentration measured using small aliquots of cells lisate.

FPLC CHROMATOGRAPHY

Bovine serum albumine (BSA) or AGE-BSA were analyzed by FPLC (Pharmacia, Uppsala, SWEDEN). Proteins were resupended in PBS then exposed to sugars as described before. After the incubation, aliquotes of 50 micrograms were injected onto a Superose Pharmacia column equilibrated with PBS without calcium and magnesium. Then elution was carried out with a 0.5 milliliter/min flow, at room temperature, and proteins peaks were detected and measured under UV recording (optical density at 214 nm). Column was calibrated with a mixture of protein molecular weight standards (Pharmacia) according to the manufacturer's instructions.

BIOINFORMATIC ANALYSIS

The bioinformatic analysis of data was performed through DAVID portal (http://david.abcc.ncifcrf.gov/). In particular proteins, identified by the UNIPROT accession number, were classified using

several parameters (e.g. functional annotation chart, KEGG analysis of pathways) to evaluate the possible role of the identified proteins in the HMGB1-interactome. Moreover, to confirm the proteomic results, data from microarray dataset of melanoma patients were analyzed through Gene Expression Omnibus (**GEO, http://www.ncbi.nlm.nih.gov/gds**).

STATISTICAL ANALYSIS

All experiments were repeated three times, and the results are expressed as the mean ± SD (standard deviation) of three different determinations. Data were analyzed by the t-Student test, differences were considered highly significant when $p<0.05$

RESULTS

GLYCATION OF BOVINE SERUM ALBUMIN

Glycated-BSA (AGE-BSA) and non glycated BSA (BSA) were prepare under sterile conditions as above mentioned, and the AGE-products have been characterized: AGE production was assessed by fluorescence and protein concentration by Bio-Rad protein assay.

AGEs products are chemically heterogeneous, but out of ~80% are characterized by specific fluorescence, so that fluorescent AGEs could be measured by spectrofluorimetric analysis modified by Sampathkumar **(Sampathkumar et al. 2005)**, which provides the measure of emission intensity at 430 nm, under excitation at 370 nm. A single volume (80 µl) of samples of BSA and AGE-BSA incubated *in vitro*, was analyzed.

The fluorescent AGE formation was related to the time of exposure to glycating sugar in a fluorescent measurements.

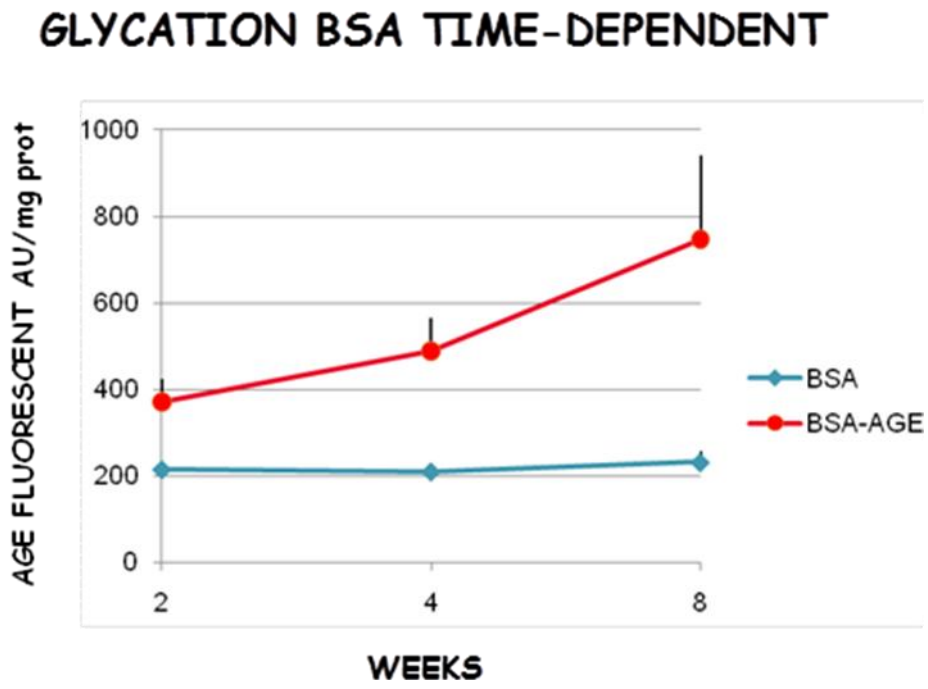


FIGURE 6: Fluorescent AGE levels (Arbitrary Unit/mg of proteins) in a BSA (4mg/ml) after 2, 4 and 8 weeks of incubation without D-glucose (BSA) and with a D-Glucose 500 mM (BSA-AGE). Data are expressed as means ± SD.

Preparations obtained with D-Glucose 500 mM for 8 weeks of incubation were found to have the greatest amount of fluorescent AGE and, for this reason, have been used for in vitro experiments. Further, glycated-BSA and BSA were also analyzed by size exclusion chromatography (FPLC), **(FIGURE 7).**

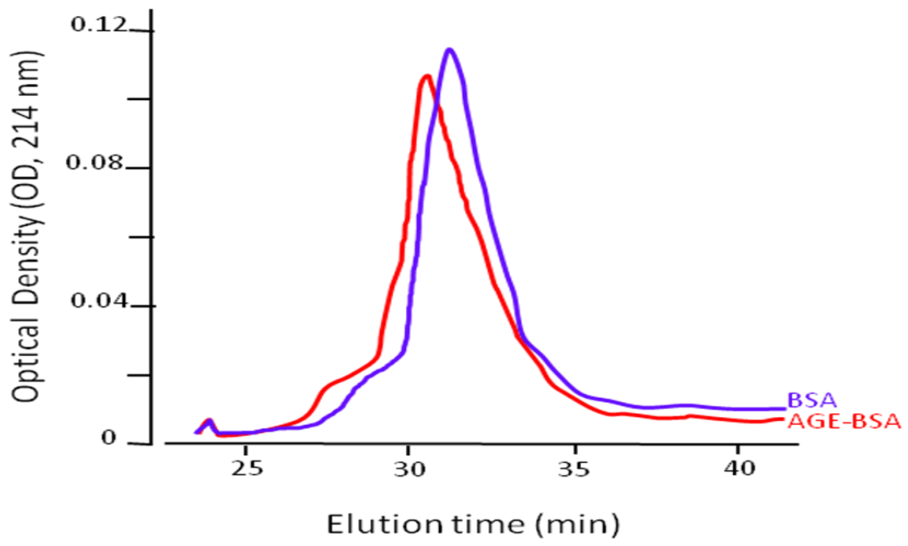


FIGURE 7: Chromatographic analysis of BSA and AGE-BSA

The chromatographic profiles confirmed that AGE-BSA (i.e. BSA incubated with glucose) was really modified by the glucose treatment, leading to the formation of complexes with a larger molecular weight when compared to control BSA.

CD26 INHIBITS HMGB1-INDUCED ENDOTHELIAL CELL MIGRATION

CD26 is a 110-kDa cell surface glycoprotein which also exists as a soluble form circulating in plasma. CD26 selectively removes the N-terminal dipeptides with either L-proline or L-alanine at the

penultimate position from a variety of chemokines, growth factors and neuropeptides. Proteolytic activity leads to inactivation or degradation of these peptides. Both glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are endogenous physiological substrates for CD26, and chemical inhibition of CD26 activity, results in increased levels of intact bioactive GIP and GLP-1 and glucose tolerance. At present, CD26 inhibitors are used as glucose-lowering agents in the treatment of type 2 diabetes **(Dicembrini et al. 2011).**

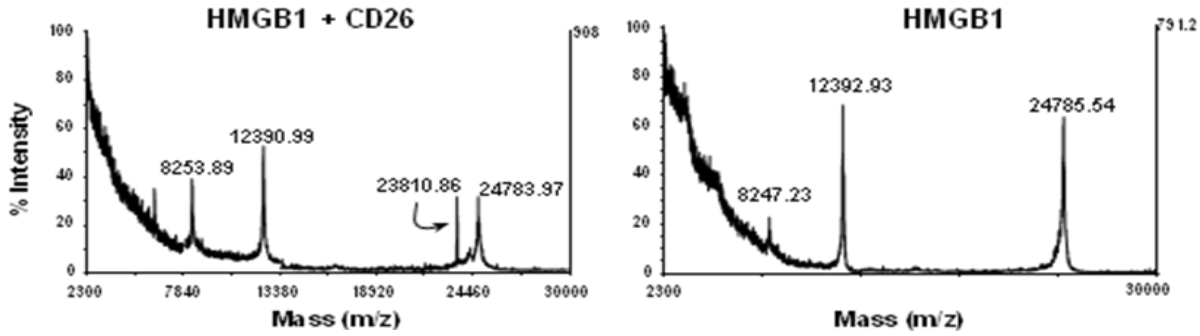
Our data support recent observation from Takasawa showing that inhibition of CD26 enzymatic activity may affect endothelial cell functions including endothelial cell proliferation and ex vivo neovascularization following TNF-a or IL-1b stimulation **(Takasawa et al. 2010).**

The impairment of angiogenesis in diabetes is well documented **(Facchiano et al. 2006)**, however molecular defects involved in diabetes-related vascular defects so far have remained elusive and growth factor inactivation as well as increased production of angiogenesis inhibitors, may account for these defects.

Migration is a key process required for angiogenesis and HMGB1 display chemotactic activity on endothelial cells **(Mitola et al. 2006; Schlueter et al. 2005; De Mori et al. 2007)**.

To investigate whether CD26 affected the HMGB1-mediated endothelial cell migration, the chetomotactic activity of intact and CD26-cleaved HMGB1 were compared in vitro on HUVEC cells.

Since the structural analysis of HMGB1 suggest that it may contain potential CD26 cleavage sites we determined whether HMGB1 may be a substrate for CD26 and, eventually, whether CD26-mediated cleavage may alter its angiogenic activity **(Marchetti et al. 2011)**.



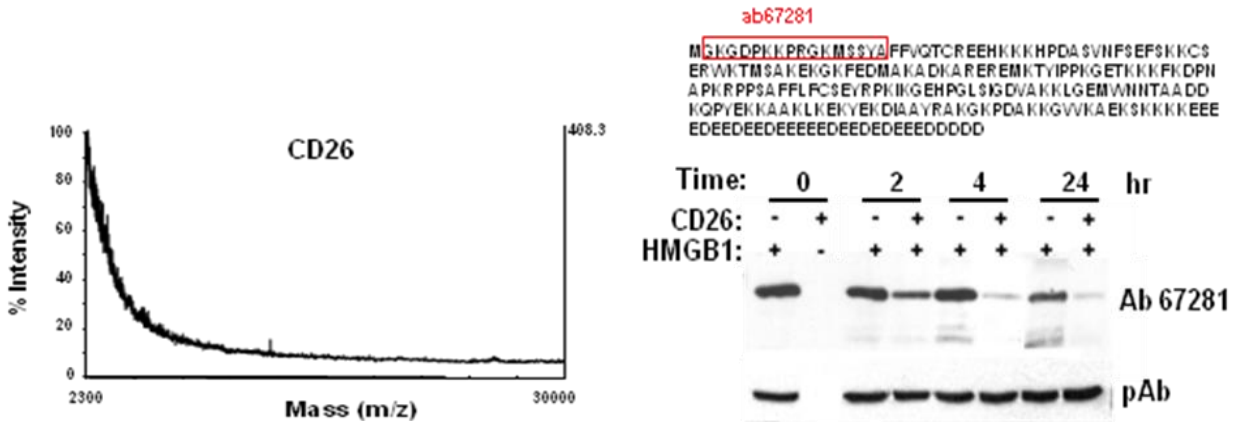


FIGURE 8: Purified CD26 cleaves HMGB1.
MALDI-TOF analysis of HMGB1. Upper pane: HMGB1 protein sequence. The red box indicates the peptide recognized by the indicated antibody. Lower panel: Western blot analysis of HMGB1 (5 μg) incubated with CD26 (0.5 μg) for the indicated time points. The same filter was probed with the anti-HMGB1 antibody that recognizes the N-terminal portion of the protein.

THE EFFECTS OF AGE ON RAT RETINAS

The *ex vivo* studies were performed on retinal tissue cultures explanted from adult rats and the *in vitro* studies were performed on retinal tissue.

Retinal tissue were exposed for 6-48 hours to high glucose (HG) levels (30 mM D-glucose), to reproduce short term effects of elevated glucose concentrations, or *in vitro* glycated Bovine Serum

Albumin (BSA) (AGE-BSA: 200µg/ml), to reproduce long term effects of elevated glucose concentrations.

Controls included 5.5 mM D-glucose, as normal glucose (NG) concentration, 30 mM D-mannitol (M) as an iso-osmotic control, and 200µg/ml non-glycated BSA. At the end of the experimental procedures, tissue or cells were treated for protein analysis characterization.

The retinal tissue were lysed and analyzed by western blot to study a modulation to the most important AGE receptor (RAGE) in a different conditions, previously described.

Thirty µg of solubilized proteins were separated by 10% SDS-PAGE and transferred. The blot was incubated with anti-RAGE.

The effects of 24-48 hours HG and AGE-BSA stimulation on RAGE expression in retinal explants are shown in **FIGURE 9** showing a significant increase of RAGE expression upon these treatment, compared to controls.

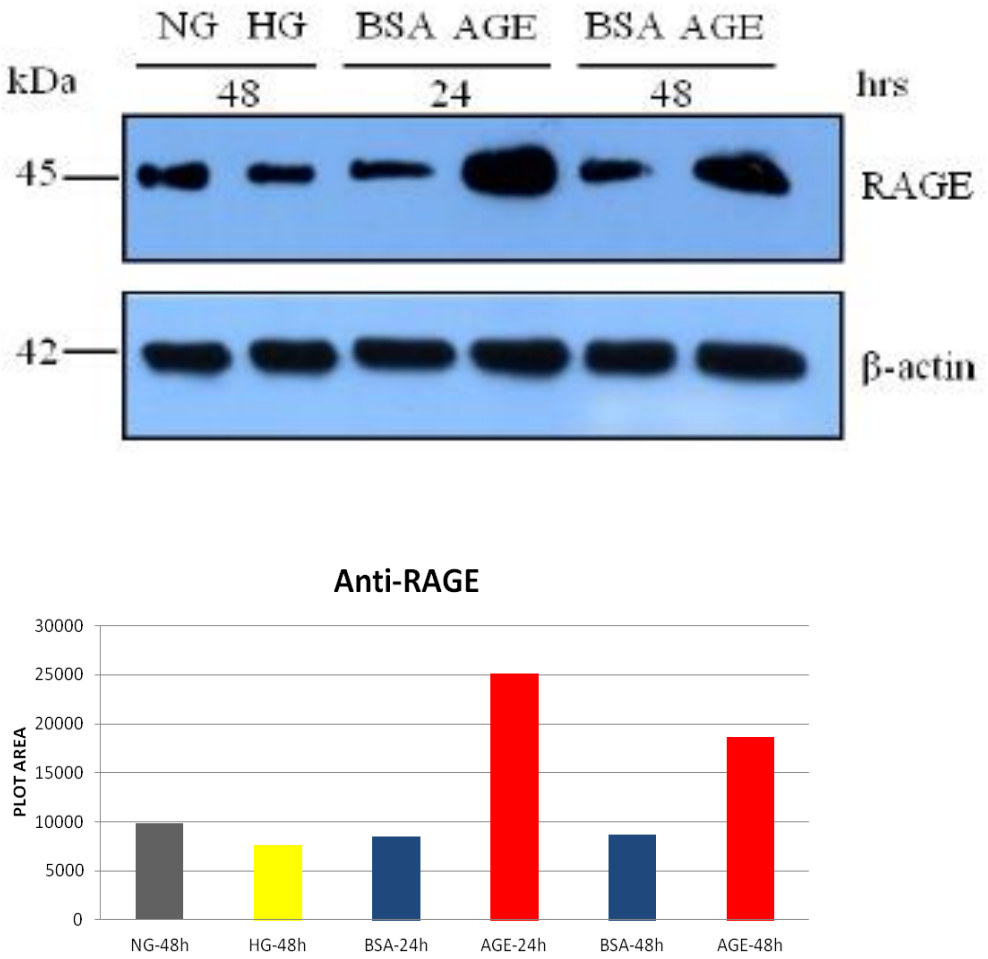


FIGURE 9: ***Effects of AGE-BSA on RAGE levels in retinal explants.*** **Rat retinal explants exposed to NG, HG, BSA and AGE-BSA for 24-48 h and analyzed by Western blot with rat anti-RAGE antibody. Solubilized proteins (30 µg) were separated by 10% SDS-PAGE. Identical samples were also run and probed with an anti-β-actin antibody to normalize the protein content.**

The same procedure was carried out to evaluate the TLR4 expression under the same HG and AGE stimulations.

Even in these experiments, the TLR4 expression in lysates of retinal explants was significantly increased compared to control (BSA) **(FIGURE 10)**.

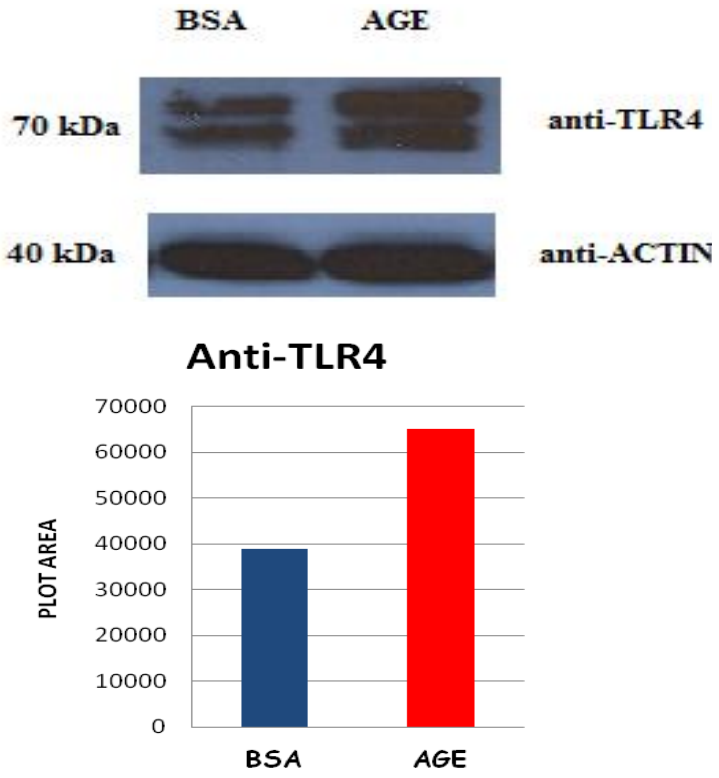


FIGURE 10: ***Effects of AGE-BSA on TLR4 levels in retinal explants*****. Rat retinal explants exposed to BSA and AGE-BSA for 24-48 h and analyzed by Western blot with rat anti-TLR4 antibody. Identical samples were also run and probed with an anti-β-actin antibody to normalize the protein content**

To perform a proteomic evaluation of retinal tissues, 30 microgram of retinal tissue protein extracts were subjected to 4-15% gradient

SDS-PAGE analysis and then stained with Coomassie G-250 BIORAD.

The whole lane of each retinal lysate (both treated and untreated) was cut and digested with trypsin and the expressed proteome subsequently identified by LC-MS/MS.

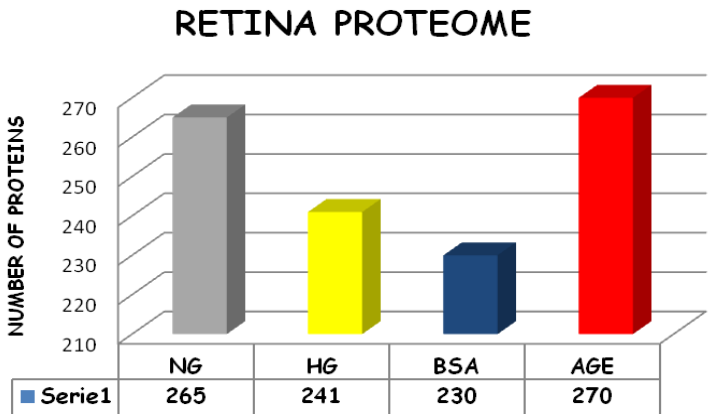


FIGURE 11: Mass spectrometry analysis of Retinal tissues; Normal Glucose, High Glucose, BSA and AGE (stimuli).

Since retinal lysates did not allow to perform a high quality interactome analysis, due to the low amount of proteins analyzed and probably to some tissue degradation during retinal preparation, an additional model was analyzed, i.e. cell cultures.

HMGB1 IN HUVEC CELLS EXPOSED TO GLUCOSE AND AGE

HUVEC were grown in the presence of BSA-AGE and AGE as described under Materials and Methods Section.

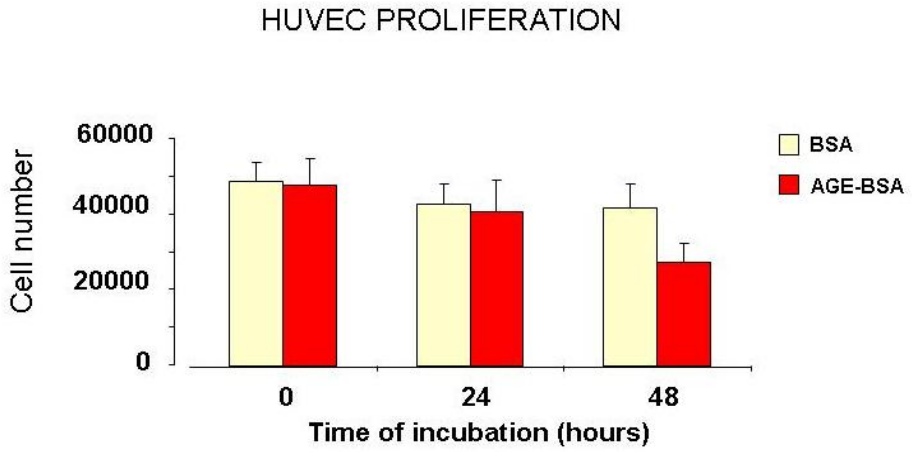


FIGURE 12: Effects of BSA-AGE and AGE on HUVEC cells proliferation rate. Data are expressed as means ± SD.

The HUVEC cell lysates were analyzed by immunoprecipitation to study the HMGB1 Interactome under our experimental condition and stumuli.

The IP was carried out using anti-HMGB1 antibody conjugated to protein A/G beads.

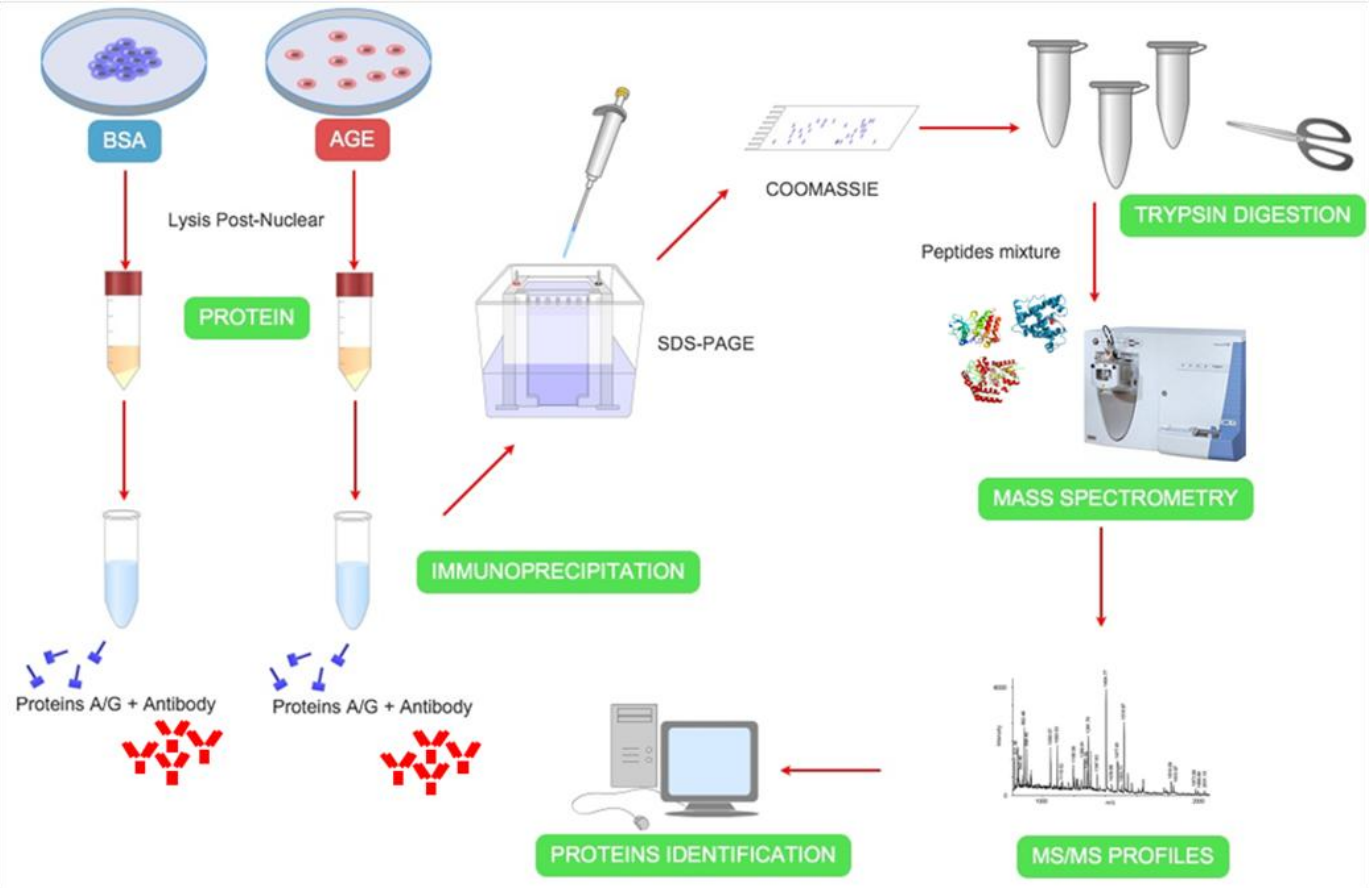


FIGURE 13: Schematization of proteomics analysis

The immunoprecipitated complex was then run on acrilammide gradient gel electrophoresis (SDS-PAGE) as described in Material and Methods.

To confirm the presence of HMGB1, the western blot was performed with anti-HMGB1 mouse monoclonal **(FIGURE 14)**.

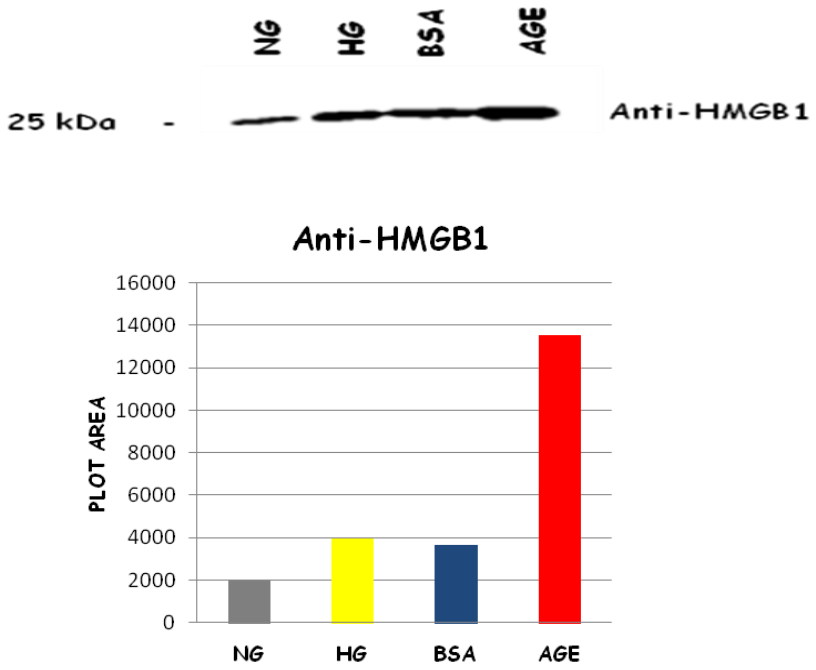


FIGURE 14: IP of HMGB1 using HUVEC cell lysates treated with (1) Normal Glucose, (2) High Glucose, (3) BSA, (4) AGE-BSA.

The increase of HMGB1 in total lysates with the different treatments was evaluated by western blot.

As seen in **FIGURE 15** the expression of HMGB1 in the cells treated with BSA-AGE is increased compared to its control BSA,

the same can be seen on the cells treated with high glucose compared to normal glucose.

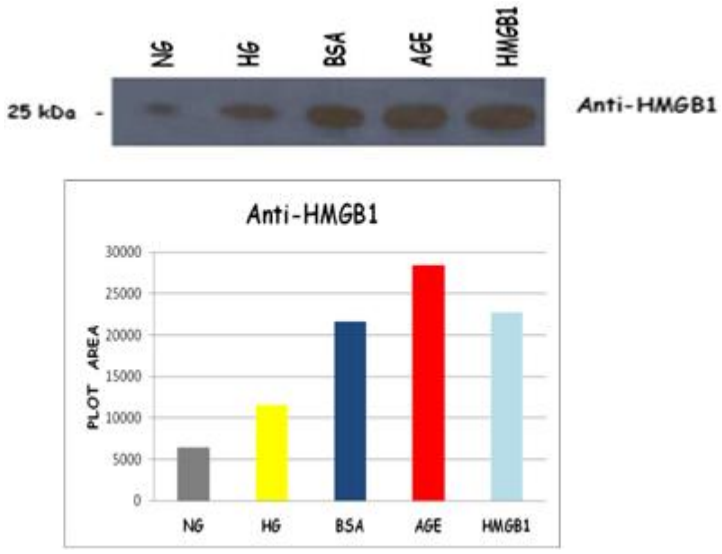


FIGURE 15: HUVEC cells with treatment with (1) Normal Glucose, (2) High Glucose, (3) BSA, (4) AGE-BSA, (5) High Mobility Group Box-1.

The proteomic analysis of protein lysates from each treatment was then performed.

The analysis was made on acrylamide gel stained with blue coomassie, each line was cut and digested with trypsin and subsequently analyzed with mass spectrometry.

Thus, we have identified the proteomes of HUVEC treated with the stimuli under study and analysis led to the result reported in **FIGURE 16.**

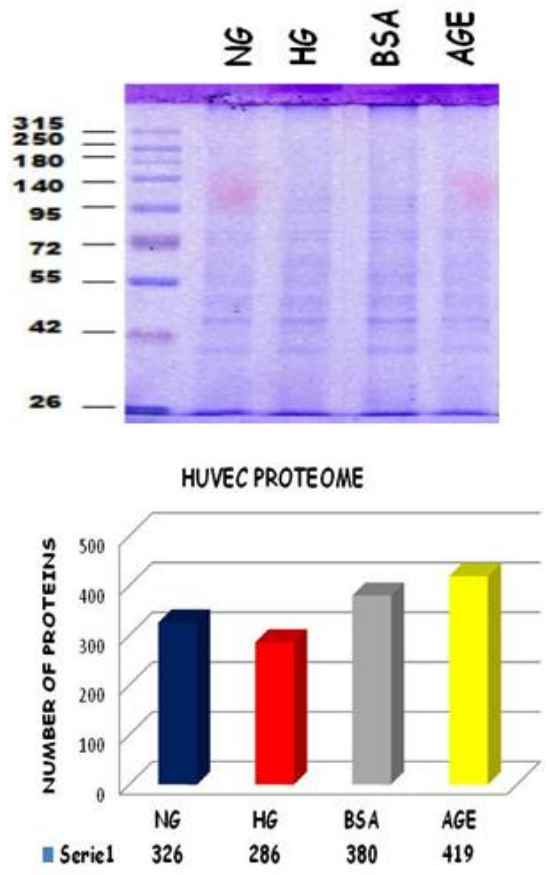


FIGURE 16: HUVEC TREATED with Normal Glucose (CTRL), High Glucose (HG), mannitol isosmotic control (M), BSA AND AGE-BSA. Equal amount of proteins were loaded for each lane (35μg).

HMGB1 IN A375 CELLS EXPOSED TO GLUCOSE AND AGE

Another cell line, human melanoma A375 cells, were also exposed to the same hyperglycaemic and AGE stimuli, to study the HMBG1 interactome.

The A375 cells were treated with AGE and its control BSA (see material and methods).

The survival of melanoma cells treated with AGE compared to melanoma cells treated with BSA was unchanged, then the cell lysates were analyzed by immunoprecipitation to study the HMGB1 interactome.

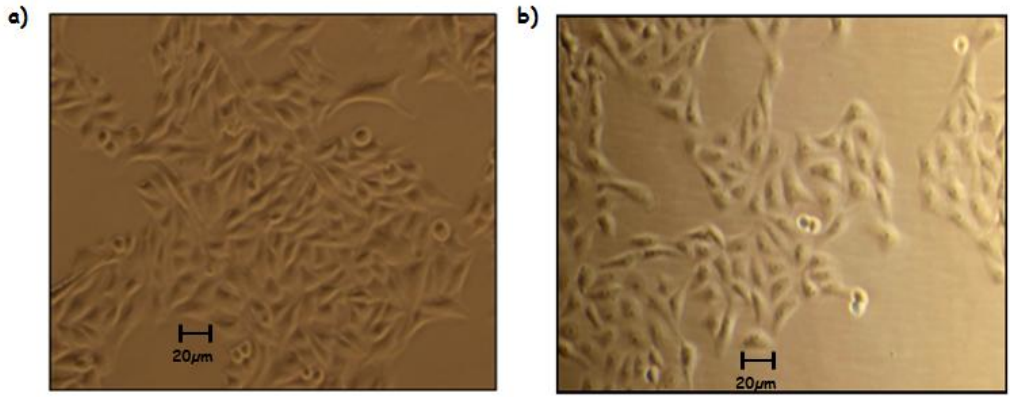


FIGURE 17: a) A375 cells treated with BSA and b) A375 cells treated with AGE (400x).

Some of the proteins identified in the total lysates treated (AGE) and their respective controls (BSA) by mass spectrometry were validated by bioinformatic analyses but further validation in ongoing by western blot.

The amount of identified proteins with mass spectrometry are described in Table X analysis on cell lysated.

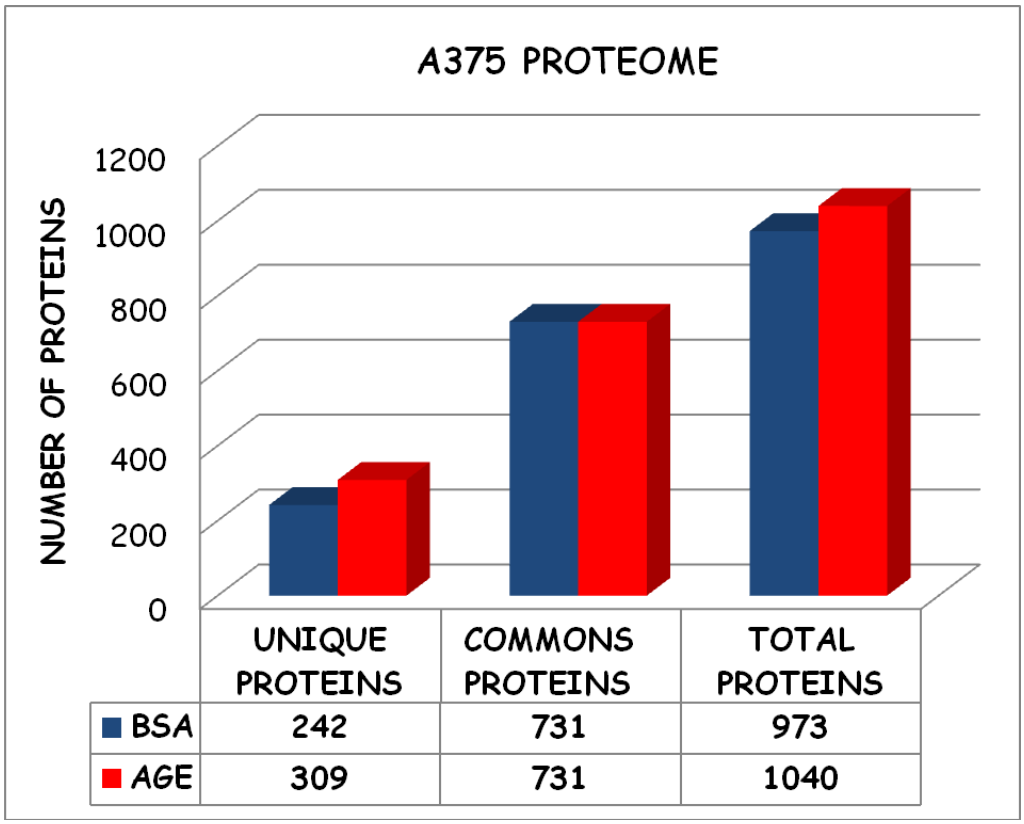


FIGURE 18: Proteomic analysis of A375 cell lines treatated with a BSA-AGE stimuli.

AGE TREATMENT INDUCES INCREASE OF RAGE AND HMGB1: STUDY OF INTERACTOME

The A375 cells were lysed and analazyed by western blot to study (see material and methods) a modulation of HMGB1 and RAGE proteins. The analysis of western blot on total lysates, showed a net

increase of HMGB1 in A375 cells treated with AGE compared to control **(FIGURE 19)**.

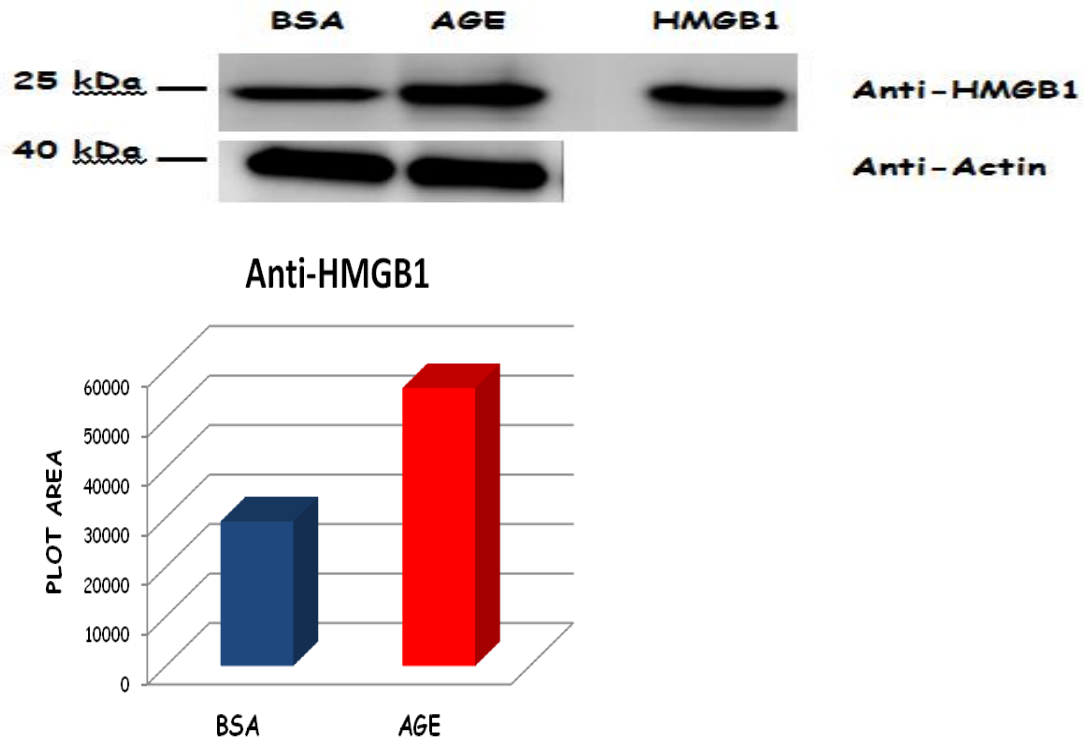


FIGURE 19: HMGB1 expression in a375 cell lysates after treatment with BSA or AGE-BSA.

The analysis of western blot on total lysates, showed a increase too of RAGE in A375 cells treated with AGE compared to control **(FIGURE 20)**.

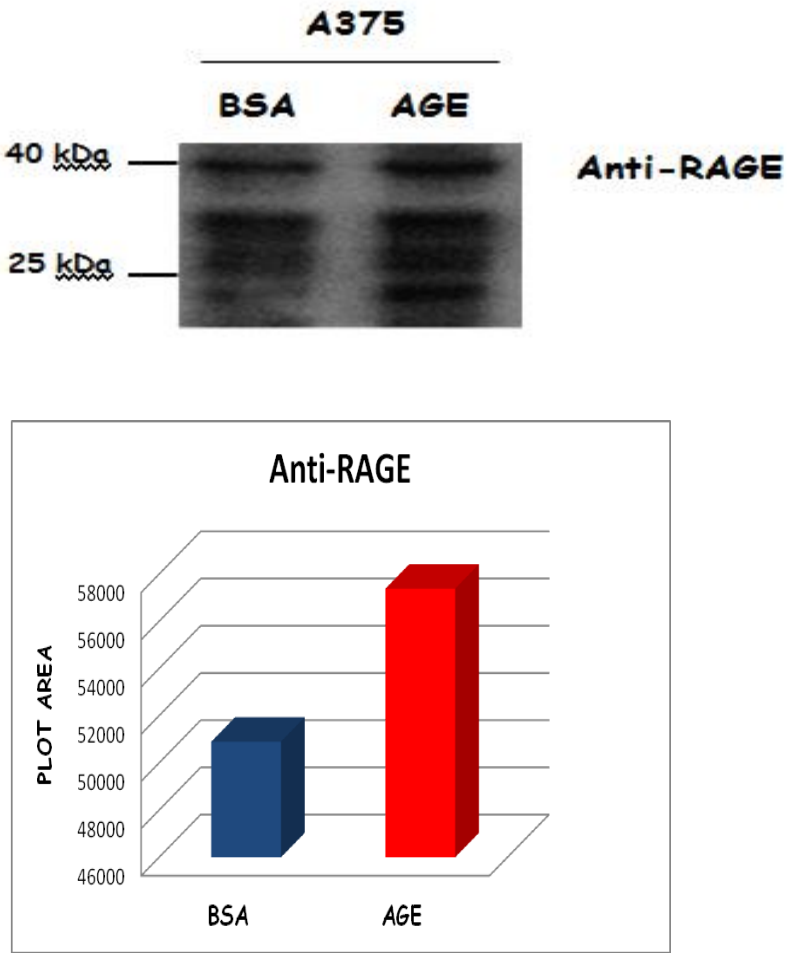


FIGURE 20: RAGE expression in A375 cell lysates after treatment with bsa or AGE-BSA.

THE AGE-INDUCED INTERACTOME TO HMGB1

The A375 cells lysated were immunoprecipitated with anti-HMGB1 antibody conjugated beads. A portion of the immunoprecipitated complexes (25% of the total sample) was electrophoretically

separated and transferred onto nitrocellulose while the remaining portion (75% of the total sample) was analyzed by LC-MS/MS. Western blot analysis of immunoprecipitated sample showed that AGE-treated cells express higher HMGB1 levels **(FIGURE 21)**.

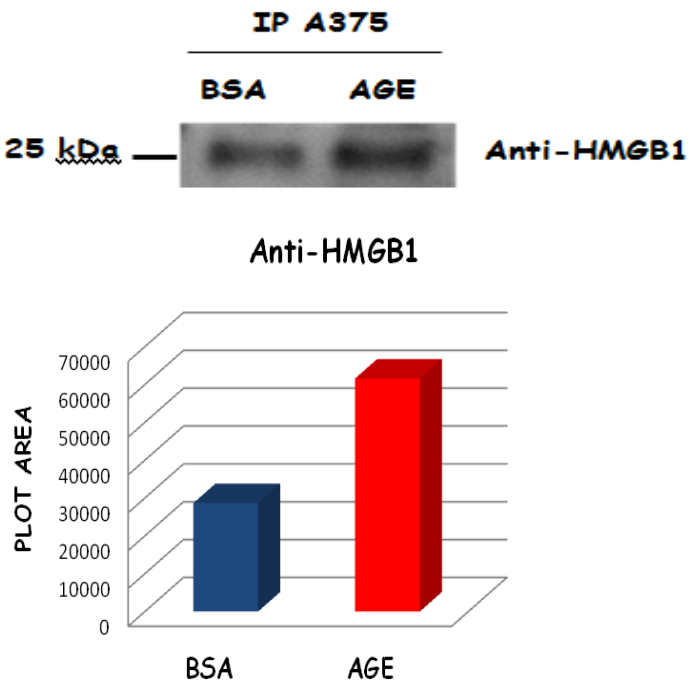


FIGURE 21: HMGB1 IP from A375 cells lysate.

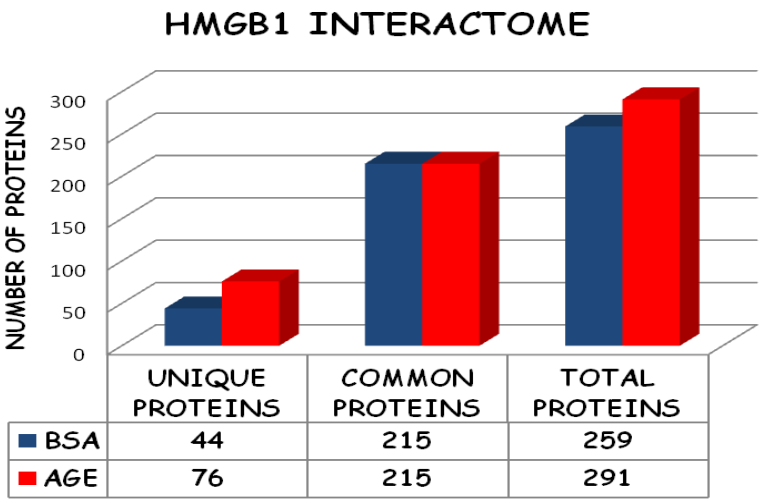


FIGURE 22: Interactome analysis of IP-HMGB1 by Mass spectrometry analysis.

	PROTEIN	ACCESSION NUMBER
1	DNJA2_ DnaJ homolog subfamily A member 2	O60884
2	MPPB_ Mitochondrial-processing peptidase subunit beta	O75439
3	LEG1_ Galectin-1	P09382
4	HMGB1_ High mobility group protein B1	P09429
5	BTF3_ Transcription factor BTF3	P20290
6	PTBP1_ Polypyrimidine tract-binding protein 1	P26599
7	CTNA1_ Catenin alpha-1	P35221
8	oST48_ Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	P39656
9	EAA1_ Excitatory amino acid transporter 1	P43003
10	MSH2_ DNA mismatch repair protein Msh2	P43246
11	XPo2_ Exportin-2	P55060
12	DHSo_ Sorbitol dehydrogenase	Q00796
13	PLoD1_ Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Q02809
14	MTX1_ Metaxin-1	Q13505
15	MoGS_ Mannosyl-oligosaccharide glucosidase	Q13724
16	IDI1_ Isopentenyl-diphosphate Delta-isomerase 1	Q13907
17	ECM29_ Proteasome-associated protein ECM29 homolog	Q5VYK3
18	PSMD1_ 26S proteasome non-ATPase regulatory subunit 1	Q99460
19	AN32E_ Acidic leucine-rich nuclear phosphoprotein 32 family member E	Q9BTT0
20	PARP4_ Poly [ADP-ribose] polymerase 4	Q9UKK3
21	PSD13_ 26S proteasome non-ATPase regulatory subunit 13	Q9UNM6

FIGURE 23: The analysis of the AGE-induced HMGB1 interactome revealed the presence of 21 "unique" proteins, confirmed by two independent experimental sets. These proteins represent the AGEs-INDUCED HMGB1 INTERACTOME PROTEINS (AGHIPs).

DISCUSSION

The principal aim of the present study is to elucidate the molecular mechanisms related to the inflammatory and angiogenic responses in human diseases like cancer and diabetes mellitus. In such diseases the alarmins are known to be key modulators of cellular response involved in the metabolic pathways of inflammation, but the molecular players involved are not yet completely understood.

The specific aims of this PhD. Thesis have been focused on a) the development of a suitable experimental model, b) production and characterization of a molecular stimulus mimicking the inflammatory/hyperglycaemic stimulus and c); the study of the HMGB1-interactome, i.e. to identify proteins that interact with HMGB1, one of the most interesting alarmins.

All the aims have been successfully carried out: In particular, a large amount of highly purified AGE-BSA was produced and characterized, by means of chromatographic analyses and functional assays. FPLC and spectrofluorescence analyses showed that AGE-BSA had higher molecular weight and fluorescence

behaviours characteristic of an AGE-modified protein, when compared to control BSA **(FIGURES 6-7)**.

Further, Multiplex measurements of cytokines (Bio-Plex assay) produced by cells exposed to this AGE-BSA showed an increased release of IL15, IL-2, MIP-1α and MIP-1β as expected when a pro-inflammatory pathway is activated **(Steel et al. 2011; De Rham et al. 2007; Coussens et al. 2002)**.

This confirmed that the AGE-BSA stimulus was able to induce the pro-inflammatory pathways described in literature, but revealed also a number of interesting responses (e.g. modulation of IL-8 and MIP-proteins) which deserves to be further investigated. The efficacy of the AGE stimulus was also confirmed by western blot analyses showing an increased expression of RAGE and TLR4 when cells were exposed to AGE **(FIGURES 9-10)**.

Using the developed cellular model, i.e. A375 melanoma cells exposed to AGE-BSA, to be compared to BSA exposed A375 cells, the HMGB1 interactome was therefore analyzed by means of immunoprecipitation methods. The efficacy and reliability of this protocol was tested and confirmed by western blot and mass

spectrometry analyses of immunoprecipitated complexes **(FIGURES 21-22)**.

The analysis of the AGE-induced HMGB1 interactome revealed the presence of 76 "unique" proteins, whose 21 proteins confirmed by two independent experimental sets. These proteins represent the AGEs-INDUCED HMGB1 INTERACTOME PROTEINS (AGHIPs)

Interestingly, some of the identified AGHIPs, i.e. galectin and PARP have been reported to be significantly involved in cancer development and progression **(Zhou X et al. 2011; János Szebeni et al. 2012;)** , confirming the efficacy of our experimental approach.

In parallel experiments carried out by means of mass spectrometry analyses, the direct interaction of HMGB1 to CD26 has been demonstrated, indicating that the increased proteolytic activity in serum diabetic patients may contribute to the degradation and/or inactivation of soluble factors involved in angiogenesis. CD26 may play a pivotal role. Consistent with this hypothesis, CD26 levels increased in high glucose cultured microvascular endothelial cells as reported in a published manuscript **(Marchetti et al. 2011)**.

The future plans of this study will be focused on the complete validation and functional characterization of the identified AGHIPs in order to identify novel molecular players of the vascular complications in DM and in angiogenic and inflammatory pathways regulation in melanoma patients.

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