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AGING IN HUMAN LIVER AND SKELETAL MUSCLE: STUDIES ON PROTEASOMES

Presentata da: Dott. ssa Morena Martucci

Coordinatore Dottorato **Relatore**

Prof. Sandro Grilli **Prof. Claudio Franceschi**

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Summary

1.INTRODUCTION

1.1 INTRACELLULAR PROTEOLYTIC SYSTEMS

In all cells, the production and degradation of proteins, commonly known as protein turnover, is a constant and crucial process for survival, tissue renewal and repair. Protein breakdown also represents a quality control mechanism, by eliminating damaged or abnormally folded proteins (Chondrogianni et al., 2012b). Additionally, continual protein degradation represents the main source of epitopes that are presented to T cells on class I and II major histocompatibility complex (MHC I and MHC II). In mammalian cells, numerous proteolytic systems take part in protein catabolism, the most important are **lysosomes, caspases, calpains and the ubiquitin-proteasome system (UPS)**.

Lysosomes are membrane-enclosed organelles that contain an array of degradative enzymes, a large variety of hydrolases and protease (e.g. cathepsins) capable of breaking down all types of biological polymers: proteins, nucleic acids, carbohydrates and lipids. Lysosomes represent the digestive system of the cell, which degrade material taken up from outside the cell by endocytosis and phagocytosis as well as obsolete components of cell itself by autophagy (Wong et al., 2010; Ciechanover et al., 2013). As several pathways terminate in the lysosome, lysosomal dysfunction has a profound impact on cell homeostasis, resulting in manifold pathological conditions, including infectious diseases, neurodegeneration and aging (Boya, 2012).

Caspases are a family of cysteine proteases that play essential roles in apoptosis, necrosis, inflammation and tissues remodeling. Each caspase is a cysteine aspartase, synthesized as inactive precursor termed procaspase. Based on kinetic data, substrate specificity and procaspase structure, caspases have been conceptually divided into initiators and effectors. Initiator caspases (e.g., CASP2, CASP8, CASP9, and CASP10) activate effector caspases in response to specific cell death signals; on the contrary effector caspases (e.g., CASP3, CASP6, CASP7) cleave various cellular proteins to trigger the apoptotic process. Dysregulation of caspases are hallmarks of many human diseases, including cancer, autoimmunity, neurodegenerative disorders and increasing evidences show that the modulation of caspase activity can confer therapeutic benefits (Howard et al., 2000).

Calpains are calcium-activated cystein proteases playing a role in various intracellular signal transduction pathways mediated by Ca^{2+}. The calpain system originally includes three proteins: two Ca^{2+}-dependent proteases, mu-calpain and m-calpain and a third polypeptide calpastatin, which is able to inhibit the two calpains. Calpains cleave target proteins at specific sites, generating large polypeptides with different physiological properties. The calpains participate in a variety of cellular processes, including remodeling of cytoskeletal/membrane attachments, different signal transduction pathways and apoptosis. Deregulated calpain activity following loss of Ca^{2+} homeostasis results in tissue damage in response to events such as myocardial infarcts, stroke, and brain trauma (Goll et al., 2003).

1.2 UPS

The UPS is the primary selective degradation system in the nucleus, cytoplasm as well as endoplasmic reticulum (ER) of eukaryotic cells, required for the turnover of myriad soluble proteins. The UPS plays two crucial roles: first of all in the maintenance of cellular homeostasis as it is responsible for the clearance of abnormal, denatured or damaged proteins, and secondly in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation and endocytosis, by the regulated degradation of short-lived proteins (Hershko et al., 1998; Chondrogianni et al., 2010).

Proteins to be degraded via UPS are firstly tagged by the covalent attachment of protein modifiers, where the most important is represented by ubiquitin, a highly conserved 76 amino acid residues polypeptide. The conjugation process is a three-step cascade mechanism which involved several multiprotein complexes. Initially, the E1 ubiquitin-activating enzyme activates ubiquitin in an ATP-dependent manner, then one of several E2 enzymes (known as ubiquitin-carrier proteins or ubiquitin-conjugating enzymes) transfers the activated ubiquitin from E1 to the substrate that is specifically bound to a member of the E3 ubiquitin-protein ligases, which can catalyze the transfer of the ubiquitin with a direct (RING domain family) or indirect (HECT domain family) reaction. Thus, the carboxyl-terminal portion of the ubiquitin is linked to an ε-NH_2 group of an internal lysine (Lys) residue in the target substrate. These repeated steps leading to produce a poly-ubiquitin (polyUb) chain that serves as a recognition marker to the downstream 26S

proteasome complex, the catalytic unit of the UPS. Proteasomal degradation has been shown to be mediated mainly by poly-ubiquitination on Lys48 of ubiquitin, but other Lys (K^{63}, K^{6}, K^{11}, K^{27}, K^{29} and K^{33}) have also been described (Saeki et al., 2009; Xu et al., 2009). Furthermore it was suggested that at least four Ub moieties are necessary for an efficient recognition and processing by the 26S proteasomes; however recent investigations show that the proteasomal proteolytic signal is far more complex and differentiated. Indeed, different kind of target protein modifications have been described including: i) homogeneous and heterogeneous Ub chains, where heterogeneous chains can be linear or single/multiply branched; ii) modification by a single Ub moiety or multiple single Ub moieties; iii) Ub modification of 'non-canonical' internal residues (Ser, Cys, Thr) in the target substrate; iv) Ub modification of the N-terminal residue of the target substrate; v) modification by Ub-like proteins (UbL) as single molecule or chain and also mixed chain made of UbL and Ub.

The ubiquitin-conjugated substrate is finally degraded to short peptides (4-25 amino acids) by the 26S proteasomes, concomitantly with release and recycle of free ubiquitin (Ciechanover et al., 2014). Considering the variety of cellular processes in which the UPS is involved, its aberrations are implicated in the pathogenesis of several diseases, including neurodegenerative diseases, malignancies, immune and inflammatory disorders.

1.2.1 PROTEASOME: STRUCTURE AND ASSEMBLY

The 26S proteasome is a highly conserved subcellular structure with multiple proteolytic activities and represents the machinery of UPS (Brooks et al., 2000; Chondrogianni et al., 2012a), localized in the cytoplasm, nucleus as well as on the ER. Its structure consists of two distinct sub-complexes: the 20S core proteasome and the regulatory 19S (or PA700) complex, that binds either to one or both ends of the 20S core (Stadtmueller et al., 2011) (**fig. 1**).

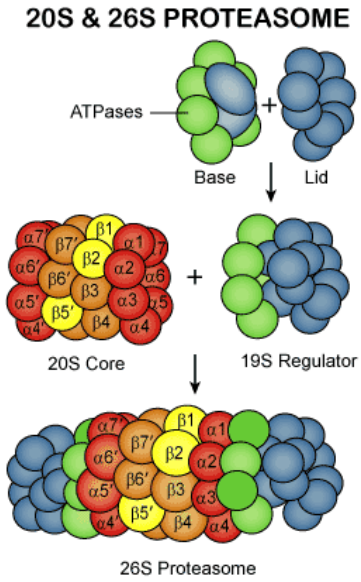


Figure 1. Structure of 20S and 26S proteasome (**www.bostonbiochem.com**).

The **20S constitutive proteasome**, a 700 kDa multisubunit enzyme, is a barrel-shape stack of four heptameric rings where two outer α-subunits rings (α1-7) embrace two central head-to-head oriented rings containing β-subunits (β1-7). The α subunits are involved in the control of the gate opening and bind regulatory complexes, whereas three of the β subunits (β1, β2, β5) harbor the active sites responsible for the proteasome hydrolyzing activities, which cleave peptide bound on the carboxyl site after, acidic (caspase-like activity), basic (trypsin-like activity), and hydrophobic (chymotrypsin-like activity) amino acids, respectively (Goldberg et al., 2007). The catalytically active residue is a single threonine located at the amino termini of the three β subunits, and characterizes the proteasome as a member of the family of **AMINO-TERMINAL NUCLEOPHILE (NTN) HYDROLASES** (Kloetzel, 2001).

The **Proteasome biogenesis** takes place at the ER and it is an accurately ordered multistep event, mediated by at least five conserved extrinsic chaperones named PAC1-4 (proteasome assembling chaperones) and UMP1/POMP (chaperone proteasome maturation protein) in mammals. The assembly of the 20S core starts with the α-ring formation and, although the precise mechanism is unknown, it is suggested that PAC3-PAC4 are required, while the role of PAC1-PAC2 is to prevent dimerization of α-rings. After the assembly of the α-ring is completed, β-subunits are incorporated onto the α-ring, initially as precursors-subunits, giving rise to half proteasome. The combination of two half-proteasomes produces pre-holoproteasomes; the maturation steps

involve the cleavage of the N-terminal propeptide from the three pairs of β catalytic subunits, thus exposing the catalytic threonine active residue. This process is assisted both by propeptides and C-terminal tails of β-subunits as well as by the assembly chaperones. Furthermore, each β subunit is recruited onto the α-ring in a defined order of β2, β3, β4, β5, β6, β1 and β7, as revealed in knockdown experiments of single β subunits in mammalian cells. The formation of mature 20S proteasomes is followed by the degradation of chaperons (Hirano et al., 2006; Fricke et al., 2007; Sahara et al., 2013).

1.2.2 PROTEASOMES REGULATORS

In order to prevent uncontrolled proteolitic degradation whitin cells, different regulators are able to monitor both recognition and degradation of proteasome substrates. The most important regulator is the **19S** (or **PA700**) **proteasomes regulator** (approximately 1 MDa), which is composed by two multisubunit sub-complexes, the lid and the base.

The lid is made of seven non-ATPase subunits (Rpn 3-9) and it is involved in the recognition and de-ubiquitination of ubiquitin-conjugated substrates, before their translocation and degradation, as well as ubiquitin recycling (Verma et al., 2002). The base directly contacts the α-rings of the 20S complex, contains six homologous ATPases (Rpt 1-6) which perform chaperone-like activities and three non-ATPase subunits (Rpn 1, 2, 10). The base allows target proteins to access the proteolytic channel, by unfolding substrates and opening the gate of the catalytic core. The 19S regulator might bind both the α-rings of the 20S core, forming a large particle named **26S proteasome**.

There are also additional regulators of the proteasome, among which the **11S complex** (or **PA28**) is the best characterized (Stadmueller et al., 2011). Three different isoforms of 11S activator are known: PA28α, PA28β, PA28γ (Rechsteiner et al., 2005). PA28α and PA28β subunits can be induced by interferon-γ and have been implicated in the production of MHC class I epitopes. It has been also proposed that PA28αβ could affect the quality of antigenic peptides produced by proteasome, by inducing changes in the structural conformation of the catalytic core (Kloetzel, 2001).

On the contrary, PA28γ is not induced by interferon-γ and it has been implicated mainly in the degradation of nuclear proteins like certain inhibitors of cyclin-dependent kinase (p21, p16,

p19). These activators have been described as involved in ubiquitin-independent degradation pathways. Moreover, as they do not show ATPase activity, it has been suggested that their substrates might be small peptides or proteins with unstructured regions, where an unfolding activity is not required.

The co-expression of 19S and 11S complexes within the same cell might give rise to **hybrid proteasomes**, that are 20S core proteasomes with both regulators at the two ends (11S-20S-19S). The exact function of hybrid proteasomes is still unknown, but likely the target substrate is recognized and bound by the 19S regulator, while the 11S might favor the release of products after proteasome hydrolysis (Tanahashi et al., 2000) or it could change its proteolytic specificities, causing a different set of peptide products (Jung et al., 2012).

It has been also recognized that certain proteins can be degraded by the 20S core proteasome in an ATP-independent manner. This is the case of ornithine decarboxylase, unfolded and defective ribosomal proteins (DRiPs), damaged and/or oxidized proteins, which are produced as consequence of oxidative stress, diseases and aging (Pickering et al., 2012b). Indeed, oxidative damage frequently leads to oxidation of aminoacid side chain and the introduction of carbonyl group, as well as conformational changes of proteins resulting in exposition of hydrophobic domains, which do not normally interact with aqueous environment. It is believed that these abnormally exposed residues act as recognition sites for the 20S proteasomes and they are able to induce conformational changes of α subunits, leading to the opening of the gate and protein degradation.

Additionally, these groups of proteins represent an important source of antigenic peptides, which are loaded on MHC class I complex and presented on cellular surface for the recognition by $CD8^+$ T-lymphocytes. Several lines of evidences suggest that a variant of the constitutive 20S proteasomes, named **immunoproteasome** (**i-proteasome**), is primarily involved in this process (Kloetzel, 2001; Strehl et al., 2005), as discussed more in detail in the next chapter. Of note, another proteasomes isoform named **thymoproteasome** exists, where a β5t replace the β5 catalytic subunit of constitutive proteasome, and it plays a role in the positive selection of $CD8^+$ T-cells (Takahama et al., 2012), but further dissertations on its patho-physiological role are beyond the aim of this thesis.

1.3. IMMUNOPROTEASOME

1.3.1 STRUCTURE AND TISSUE DISTRIBUTION

As a consequence of pro-inflammatory stimuli, such as exposure to Tumor Necrosis Factor-α (TNF-α), Interferon γ (IFN-γ) or Lipopolysaccharide (LPS), the constitutive catalytic subunits β1, β2 and β5 are replaced by inducible β-counterparts, named β1i (or low molecular mass peptide 2, LMP2), β2i (or multicatalytic endopeptidase complex-like 1, MECL-1) and β5i (or low molecular mass peptide 7, LMP7) respectively. Under these conditions, proteasome has to be synthesized *ex novo,* leading to the formation of immunoproteasome.

The two genes that encode β1i and β5i subunits are mapped in the MHC class II region, located on chromosome 6 in humans, and they are clustered with the TAP-1 and TAP-2 genes (transporter associated with antigen processing), while gene encoding the third inducible catalytic subunit β2i is found on chromosome 16 (Zhou et al., 1993). Like β1i and β5i, the expression of TAP proteins is also up-regulated by IFN-γ (Yang et al., 1992). Indeed, their promoter region contains binding sites for multiple transcription factors, including the interferon consensus sequence-2 and the gamma interferon activated sequence, which bind the dimer signal transducers and activators of transcription-1 (Stat-1) and the interferon regulatory factor-1 (IRF-1) (Chatterjee et al., 2000), the major transcription factors involved in IFN-γ signal transduction. Additionally, cAMP responsive elements and regions of consensus sequence for NF-kB binding are also present, thus suggesting that additional cytokine-independent regulation mechanisms are possible.

Taking into consideration that only the X-ray structure of murine i-proteasome has been solved (Huber et al., 2012), bioinformatic analyses showed that human constitutive and inducible proteasome subunits are characterized by an high conservation of the amino acids in the binding pocket of substrates, except for β1i, that contains two prominent substitutions that reduce the size of the binding pocket and change the overall charge of the local environment from positive (β1) to neutral. These changes could in part explain the drastic reduction in the caspase-like activity of the i-proteasome; indeed the cleavage after acidic residues, which is accomplished by the β1 constitutive subunit, is nearly abolished with the β1i (Toes et al., 2001). Additionally, i-proteasome shows an increase of the chymotrypsin-like activity, which promotes the generation of MHC class I peptides containing hydrophobic C-terminal anchors, thus favoring the production of antigenic peptides. However, it is necessary to underline that the various catalytic subunits

confer to i-proteasome different cleavage preferences and degradation rates, which in part are determined by amino acids that constitute the binding pocket, but also from the amino acidic composition of the substrate (Groll et al., 1997; Groll et al., 2002). Moreover, as above described for constitutive 20S proteasomes, the i-proteasome can bind both the 19S and PA28 regulatory complexes, which might affect its function at different extent (**fig. 2**).

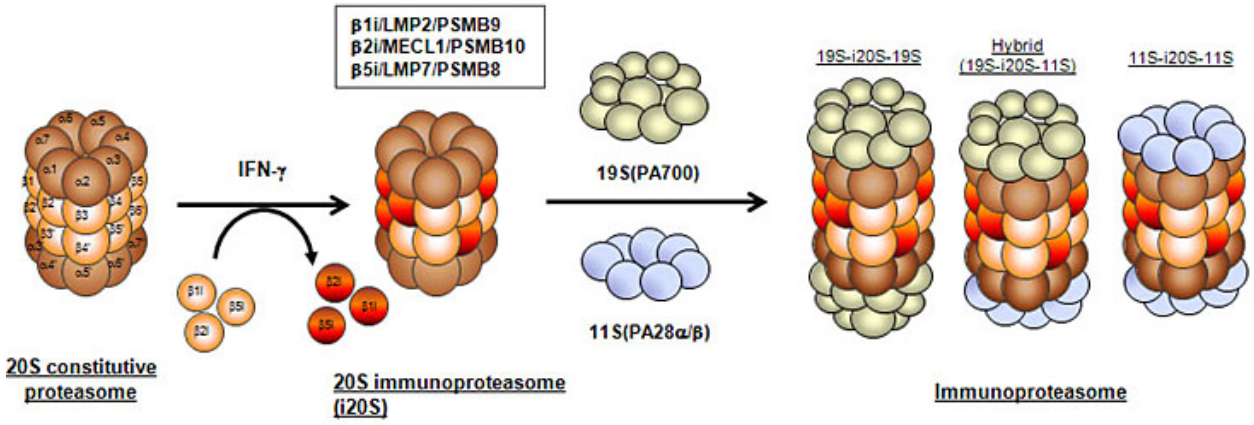


Figure 2. Inducible subunits form the 20S immunoproteasome (i20S). The mature i20S then binds to either PA700 (19S proteasome), or PA28αβ (11S proteasome), or a combination of both proteasome activators at its two ends to form three different types of immunoproteasome (Angeles et al., 2012).

Interestingly the constitutive and inducible β subunits often coexist in cells, giving rise to **intermediate types or subtypes proteasomes** (Vigneron et al., 2012). This ability of the cells to produce various subtypes of proteasomes with different catalytic properties and substrate preference has several advantages. Firstly, this plasticity allows the cell to respond to environmental condition changes and to adjust the proteolytic capacity to new challenges. Secondly, the presence of different subtypes increases the repertoire of substrates that can be degraded and of peptides eventually generated.

I-proteasomes were firstly identified in vivo in cells and organs of the immune system, such as T cells, B cells, monocytes, macrophages, dendritic cells, or medullary thymic epithelial cells (Kniepert et al., 2013). In line with its role in shaping the MHC class I-restricted peptide repertoire, it has been shown that the spleen has the highest i-proteasome expression and activity than any other tissue in rats (Noda et al., 2000). Additionally, it was long assumed that most of the tissues of non-hematopoietic origin and organs without immunological functions were normally devoid of

i-proteasome. However, over the past few years, an increasing number of studies have re-defined i-proteasome expression in a wide array of non immunological cells and organs (Ebstein et al., 2012). Indeed, the β5i inducible catalytic subunit is constitutively expressed in normal tissues as colon, liver, umbilical vein, placenta including small intestinal epithelial cells. In addition, a fully mature i-proteasome, containing the all three inducible catalytic subunits, can be purified from human kidney. Noteworthy, in both colon and liver, β5i is accompanied by the up-regulation of the two other inducible subunits β1i and MECL-1 during the course of pathologies, including Crohn's disease (Visekruna et al., 2006) and cirrhosis (Vasuri et al., 2010), probably as result of an alteration of the local inflammatory environment. Most surprisingly, i-proteasome has been reported to be expressed in uninjured immune privileged tissues, such as the retina (photoreceptors and neurons in the outer layer of the retina) (Ferrington et al., 2008) and brain (synapse and Purkinje cells) (Mishto et al., 2006a; Diaz-Hernandez et al., 2003). Additionally, earlier studies in humans have suggested that i-proteasomes are absent in neurons under normal conditions, but they can be induced during neurodegenerative diseases such as Alzheimer (Mishto et al., 2006a), Multiple Sclerosis (Mishto et al., 2010) and Huntington (Diaz-Hernandes et al., 2003).

The nature of the stimuli leading to the basal and persistent expression of one or more of the three inducible catalytic subunits even under healthy conditions remains unclear. None of cell types above mentioned is a strong producer of IFN-γ, thereby excluding the possibility that the formation of i-proteasome is driven by permanent autocrine stimulation. The prolonged expression of β1i, β5i and MECL-1 subunits in these tissues might rely on the secretion of pro-inflammatory cytokines by neighboring cells such as resident macrophages and infiltrating T cells or NK cells, which secrete high levels of IFN-γ during pathological condition, but it is quite unlike in uninjured environment. Therefore, it is also possible that the constitutive expression of i-proteasome subunits in these non-immune tissues may reflect a permanent activation of intracellular signaling pathways (Ebstein et al., 2012), which deserve further investigations.

1.3.2 FUNCTIONS: ROLE IN MHC CLASS I ANTIGEN PROCESSING VS NON IMMUNE FUNCTIONS

Proteasomes efficiently play the first step in MHC class I-restricted antigen presentation by providing the correct C-terminus of antigenic peptides, which are generated mainly by the chymotrypsin-like activity of the β5 constitutive catalytic subunit and enhanced in presence of β5i and β1i inducible catalytic subunits. However, studies on Knock-out mice for i-proteasome subunits did not show an evident phenotype. Indeed, mice deficient in β5i have exhibited a modest reduction in the surface expression of MHC class I molecules and reduced levels of $CD8^+$ T cells (Fehling et al., 1994), while deficiency in β1i seems to determine a decrease in the number of $CD8^+$ T cells and a reduced response to different epitopes (Basler et al., 2006). Even triple-deficient mice (knock-out for all three inducible catalytic subunits) were viable, fertile and immunocompetent (Kincaid et al., 2011), suggesting that although there were differences in knock-out mice regarding antigen processing and in the response toward specific antigens, there was no evidence for a global deficit in immune function.

Interestingly recent findings suggest that, beyond the antigen presentation, the i-proteasome is involved in the regulation of different biological processes, such as oxidative stress and cell cycle progression, as well as cytokine production (Ebstein et al., 2012). Different studies suggested a role of the i-proteasome in maintaining cellular homeostasis during IFN-mediated oxidative stress, by removal oxidized proteins and preventing the accumulation of harmful protein aggregates (Seifert et al., 2010, Pickering et al., 2012a). Accordingly, i-proteasome is significantly up-regulated in the central nervous system in response to acute injury (Hussong et al., 2010) and in cultured cells exposed to oxidative stress (Ding et al., 2003). Increased levels of oxidized proteins in the brain and liver of β1i knock-out mice (Ding et al., 2006) and a greater sensitivity to oxidative stress exhibited by retinal pigmented epithelial cells of double knock-out mice in β5i and MECL-1 subunits, support a role of i-proteasome in protecting against oxidative damage (Ferrington et al., 2008). In murine embryonic stem cells, the removal of oxidized proteins occurs during cell maturation phase and Hernebring and co-workers showed that this phase of differentiation is accompanied by the induction of genes encoding for the PA28αβ proteasome activator, subunits of the i-proteasome and TNF-α, with the assembly of mature forms of PA28-20S and an increase of proteasome activity (Hernebring et al., 2013). It has also been reported, in human embryonic stem cells, an enhanced expression of genes encoding for the inducible subunits of the 20S, which are down-regulated during differentiation, suggesting a role of i-

proteasome in the degradation of cell cycle regulators and/or differentiation inducing factors (Atkinson et al., 2012). The link between i-proteasome and differentiation was also found in patients with a missense mutation in β5i gene, who suffered from auto-inflammatory response, characterized by impaired adipocyte differentiation and increased level of IL-6 (Kitamura et al., 2011). This latter study also added new insights on the involvement of i-proteasome in regulation of pro-inflammatory cytokines during cellular stress response, by acting on key signaling pathways, the most important of which is represented by NF-kB (Maldonado et al., 2013).

1.4 CIRCULATING PROTEASOMES

Although proteasomes are intracellular enzymes, they have been detected in extracellular spaces and fluids, e.g. extracellular alveolar space, blood serum and plasma as well as cerebrospinal fluid, and they were defined extracellular or circulating proteasomes (c-proteasomes). Taking into account that intact and proteolytically active c-proteasomes are detectable even in healthy subjects, their incidence and relevance has been studied in several pathological conditions (Sixt et al., 2008). Wada and colleagues (Wada et al., 1993) for the first time have measured c-proteasomes concentration in serum samples of patients with hematologic malignancies and it was positively correlated to the tumor burden. Interdependency between the stage of malignancy and the level of c-proteasomes was also detected in patients with solid tumors and cancer-predisposing diseases (e.g. liver cirrhosis) as well as in several autoimmune diseases (e.g. systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis) (Egerer et al., 2002). In general these studies suggest that the amount of c-proteasomes might be a marker of disease activity, for which the improvement in the clinical course usually embraces a reduction of c-proteasomes levels. Additionally, c-proteasomes concentration has been proposed as independent prognostic factor for survival in multiple myeloma (Jakob et al., 2007). Noteworthy, to date, changes in the levels of c- proteasomes during human aging have not yet been investigated.

The origin of the circulating proteasomes is still a matter of debate. Zoeger and co-workers (Zoeger et al., 2006) have excluded that c-proteasomes may have origin from the major cell population of the blood (erythrocytes, monocytes, polymorphonuclear cells, B cells, T-

lymphocytes and platelets). Indeed, c-proteasomes isolated from plasma of healthy subjects and patients with autoimmune disease exhibited different profiles from those of blood cells. It has also been proposed that c-proteasomes might be released into the blood plasma from apoptotic or damaged cells, but this hypothesis explains only the elevated concentrations of c-proteasomes measured in patients suffering from diseases characterized by a significant increased of cell death. In fact, immunological activity, rather than the cellular damage, seems to be the cause of increased of c-proteasomes levels in the case of sepsis and severe injury (Roth et al., 2005). Surprisingly, active mechanisms of release of proteasomes have been suggested by immunoelectron microscopy analysis, which revealed vesicles containing proteasomes, in merge with the outer membrane of the choroid plexus cells (Mueller et al., 2012). Furthermore, proteasomes have also been detected in exosomes of mesenchymal stem cells, this suggests that cells can secrete proteasome via the exosome pathway (Lai et al., 2012).

1.5 PROTEASOMES AND DISEASES

Taking into account that the UPS is involved in the regulation of basic cellular processes including cycle and proliferation cell, gene transcription, apoptosis, signal transduction, antigen processing (**fig. 3**), it is not unexpected that its impairment might lead to pathological consequences (Ferrington et al., 2012; Lecker et al., 2006).

Actually, altered proteasomes activity and expression, and/or accumulation of ubiquitin-conjugated proteins have been reported in multiple diseases, including neurodegenerative disorders such as Alzheimer's disease (Mishto et al., 2006a), Parkinson's disease (Um et al., 2010) and Huntington's disease (Diaz-Hernandez et al., 2003). In these disorders, oxidative stress and inflammation play a pivotal role, but whether changes in proteasomes expression and activity can be considered a consequence of altered cellular conditions or part of mechanisms involved in disease onset, is still a matter of debate.

Proteasomes also play a role in cancer progression since they are involved in the degradation of a myriad of proteins such as oncogenes, tumor suppressors, transcription factors and signaling molecules, including the inhibitor of nuclear factor κB (IκB), the tumor suppressor p53, inhibitors of kinases p21 and p27 and of the proapoptotic protein Bax, among others

(Hershko, 2005). Accordingly, in the last decade, proteasomes have been considered a suitable target for cancer therapeutic treatment and to this purpose several compounds have been developed, tested in vitro and two of these have been approved for therapy in humans (Orlowski et al., 2008). The first one, named Bortezomib, was approved for treating of relapsed multiple myeloma and of mantle cell lymphoma, while the second one, named Carfilzomib, was approved for use in patients with multiple myeloma who received at least two prior therapies, including Bortezomib.

Further, the deregulation of the i-proteasome has been associated with various auto-inflammatory diseases, as rheumatoid arthritis (Muchamuel et al., 2009) and Nakajo-Nishimura syndrome (Kitamura et al., 2011).

Finally, despite the increased concentration of i-proteasome subunits found in patients suffering from myofibrillar myopathy and inclusion body myositis (Ferrer et al., 2004), the role of proteasomes and in particular of i-proteasome in muscle wasting and sarcopenia has to be fully investigated yet.

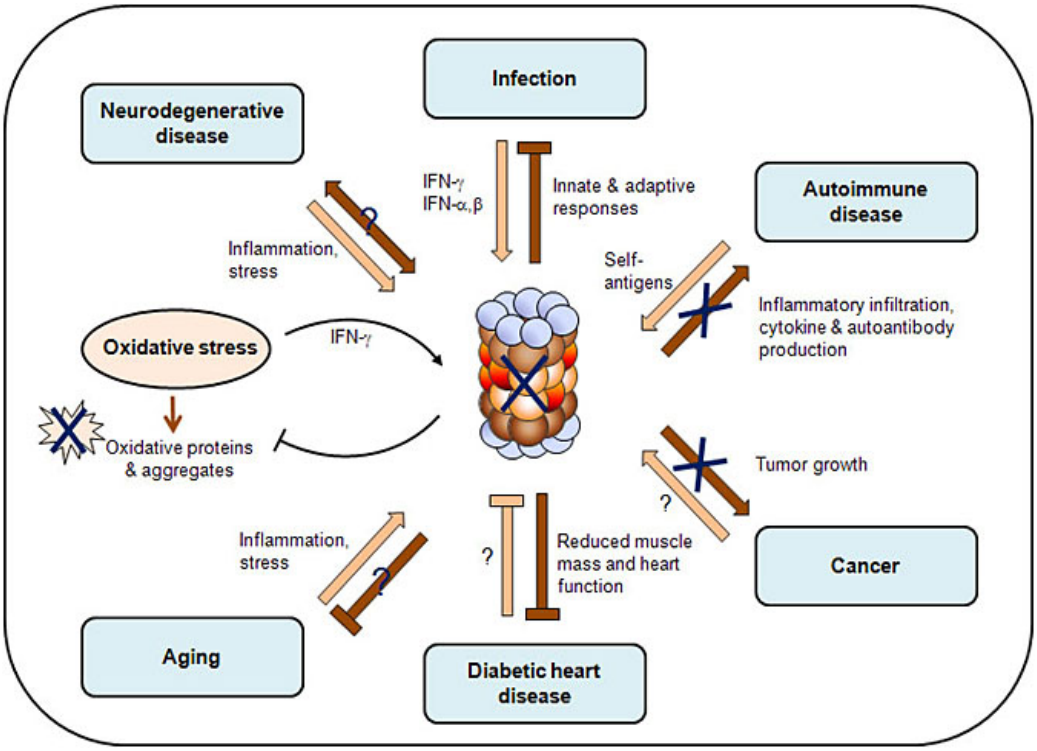


Figure 3. Proteasomes and disease. Deregulation of the immunoproteasome has been associated with various clinical disorders, including cancer, autoimmune disease, neurodegenerative disease, heart disease, aging, and infection, as well as the conditions of oxidative stress (Angeles et al., 2012).

1.6 HUMAN AGING AND PROTEASOMES

It is now recognized that human aging is a complex phenotype, resulting from the continuous adaptation of the body to unrepaired molecular and cellular damages, caused by a variety of external and internal agents. Additionally, genetics, stochasticity and environmental elements contribute at different degree to determine the aging phenotype.

At the molecular level, the maintenance of homeostasis is a key feature that determines the organism lifespan and it is influenced by several intracellular functions, including an efficient protein degradation. During aging the accumulation of damaged proteins is observed in different cell types (Mishto et al., 2003, Grune et al., 2004). In particular, levels of oxidized proteins are reported to increase significantly with age in human dermal fibroblasts, keratinocytes, erythrocytes and brain (Petropoulos et al., 2000, Levine et al., 2001). Such an age-dependent alteration could be related to a decline of proteasome activities, that a number of studies have observed in several human tissues (lens, lymphocytes and epidermis cells) as well as in other mammals such as mice, rats and cattle (liver, spinal cord, lens, heart and retina) (Chondrogianni et al., 2008). Different theories have been developed to explain such a decline. It has been proposed that the decrease of proteasome activity is a result of the inhibiting effect of protein aggregates accumulated during aging (Grune et al.,2004), or of a reduced rate of proteasomes biosynthesis and assembly, as observed in peripheral blood T lymphocytes and in epidermis cells (Ponnappan et al., 2007, Bulteau et al., 2000). Accordingly, an age-associated decrease in proteasome content and activities has been described in human dermal fibroblasts, where a restoration of normal levels of proteasome reduces cellular aging markers (Hwang et al., 2007). Additionally, it has been recently shown that naked mole-rats, a species of long-living rodents, possess an high activity of proteasomes, which might lead to the efficient removal of stress-damaged proteins and contribute to reach the extreme lifespan of the organism (Rodriguez et al., 2012). At last, proteasome activity could also be inhibited by an increased number of post-translational modifications of its subunits as detected in human epidermis cells (Bulteau et al., 2000) or by the inhibition of other regulatory molecules of the UPS (Ferrington et al., 2005). Several studies demonstrated that all of these mechanisms could occur during aging and affect proteasome activity. Likely, the impact of each single mechanism on proteasome function could vary between different tissues and cell types, as well as among multiple isoforms of proteasomes.

Several studies have been published on the expression of i-proteasome subunits in different tissues and cell types during aging. A decline of i-subunits in human T lymphocytes (Ponnappan et al., 2007) and an inability of senescent human fibroblasts to induce i-subunits expression following INF-γ treatment (Stratford et al., 2006) have been described. On the contrary, an increased expression of β1i and β5i i-subunits has been detected in human hippocampus of adults, as well as in brain areas of old non-human primates, without age-related modification in total 20S proteasome content. A role for the immunoproteasomes in the aging process is also in agreement with the theory of inflammaging, originally proposed by Franceschi and co-workers (Franceschi et al., 2000), which postulates that aging is at least in part triggered by an increased inflammation. Indeed, many studies indicate that old people are characterized by increased levels of inflammatory markers such as cytokines, chemokines, reactive oxygen species (ROS), as well as decreased levels of antioxidant enzymes such as superoxide dismutase (SOD) and phase 1 detoxifying enzymes (Franceschi et al., 2007, Cevenini et al., 2013). According to this theory, the persistence of inflammatory stimuli over time represents the biological background favoring susceptibility to age-related diseases/disabilities.

In the last two sections we will focus specifically on liver and skeletal muscle aging (Grizzi et al., 2013; Sheedfar et al., 2013) and the role of proteasomes.

1.6.1 HUMAN AGING AND PROTEASOMES: THE LIVER

Aging is associated with a variety of morphological changes in the liver, but their underlying mechanisms are still unclear. The liver progressively shrinks by 20-40% during the course of human life, with a concomitant age-related decrease in its volume. The classic gross appearance of the liver in the elderly is known as “brown atrophy”, and the brown color is due to an accumulation of highly oxidized insoluble proteins, known as lipofuscin, into hepatocytes. Other sub-cellular changes with age in the hepatocyte involve a marked reduction of smooth endoplasmic reticulum surface, which correlates with decreased hepatic microsomal protein concentrations and enzymatic activity, including glucose-6-phosphatase. Furthermore, the human liver tends to develop macro and polyploid hepatocytes with increased nuclei and nucleoli during aging. Elderly secrete less bile acid, have an increased biliary cholesterol levels, and show an increased oxidative stress that is mainly attributable to a reduced capacity to eliminate metabolically generated

superoxide radicals. Aging of the liver is also associated with impaired metabolism of drugs, adverse drug interactions, and susceptibility to toxins. Along with the considerable growth in Western societies of relatively healthy elderly populations, it is inevitable that there will also be an increase in the number of elderly people with chronic liver diseases (mainly hepatitis C virus (HCV)-related cirrhosis, alcoholic cirrhosis and HCC) (Grizzi et al., 2013).

It is indubitable that interest in the role of aging in the sphere of hepatology has increased, especially with the recent recognition of the critical importance of age in determining the clinical outcome in (HCV)-related chronic hepatitis and the influence of donor age on graft survival after liver transplantation (Grizzi et al., 2013). Although the human liver is not unscathed by the process of aging, as above described, its changes are minor compared with other organism systems. Indeed, studies in humans and in experimental model suggest that, in comparison with other organs, the liver ages fairly well (Schmucker et al., 2011).

Additionally, orthotopic liver transplants where the age of the donor is considerably higher than that of the recipient have notably increased in the last years and clinical data indicate that liver graft from aged subjects has, in specific conditions, function and duration comparable to those achievable with liver grafts from young donors (Cescon et al., 2003; Gastaca et al., 2005; Cescon et al., 2008; Schmeding et al., 2010). This evidence seems to indicate for the liver a peculiar aging pattern, where the biological age does not correspond to the chronological age of the organ/donor, and indirectly confirms its capacity to rejuvenate when in presence of an appropriate microenvironment (Conboy et al., 2005). The molecular mechanisms involved in this peculiar aging pattern are still largely unknown, but efficient protein degradation machineries might play an important role by contributing to the maintenance of cellular homeostasis (Chondrogianni et al., 2010; Koga et al., 2011).

At present, despite the pivotal role of proteasomes in physiological as well as pathological conditions, no data are available on the age-related modifications of proteasomes in human liver and controversial results emerged in rodents. In truth, in liver of aged mice and rats all or some of proteasome activities have been found to decrease (Shibatani et al., 1996; Conconi et al. 1996; Breusing et al., 2009; Hayashi et al., 1998; Rodriguez et al., 2010; Rodriguez et al., 2012) or decrease temporarily before increasing again (Keller et al., 2000; Petersen et al., 2010) or be preserved (Ding et al., 2006; Huber et al., 2009). Dasuri and colleagues showed as aging acts on proteasome remodeling in the liver, including decrease in proteasome activity, increase in

proteasome biogenesis, alteration of proteasomes subunits and regulators (Dasuri et al., 2009). Moreover, very recently, a shift toward proteasomes containing inducible subunits in aged rat liver has also been described (Gohlke et al., 2013).

These data underline a dynamic nature of the proteasome and suggest that the relationship between proteasome composition/biogenesis and proteasome functionality in tissues is extremely complex (Dasuri et al., 2009).

Studies performed in the laboratory of Professor Franceschi, in collaboration with the Surgery Unit of Liver Transplantation and multi organ and Pathological Anatomy ''Addarii'' of Hospital S. Orsola in Bologna, showed the absence of i-proteasome in hepatocytes during fetal life, but its cytoplasmic induction occurs already at the third month of extrauterine life (Vasuri et al, 2010). Whereas after birth the most important phenomenon is the bacterial colonization of the gastrointestinal tract, this event could trigger pro-inflammatory stimuli responsible for the basal expression of the i-proteasome. In this aspect, the liver is different from other organs we studied, such as the brain, where the i-proteasome is absent in healthy young subjects (Mishto et al., 2010).

1.6.2 HUMAN AGING AND PROTEASOMES: THE SKELETAL MUSCLE

During aging, humans and other mammals undergo a physiological process named skeletal muscle wasting, characterized by a reduction in muscle mass, due to a loss of net protein content in myofibers. Several factors seem to be involved, including a reduction in protein synthesis and an increase in intracellular proteolysis, due to activation of different pathways, including the UPS (Sandri, 2013; Murton et al., 2008), among others.

Muscle wasting occurs in a variety of physiological or pathological circumstances, such as inactivity or muscle disuse, fasting and several diseases as cancer, renal failure or trauma. Since skeletal muscle is the major protein reservoir of the body, muscle wasting has beneficial effect in its initial stage, in fact it provides the organism with an ample pool of free amino acids that can be reused for energy production or protein synthesis in vital organs. However, in a variety of pathologies like cancer, sepsis and diabetes, this catabolic state is maintained over a long period of time and can become life-threatening (Mitch et al., 1996). The early step in muscle wasting seems

be the dissociation or at least the destabilization of the myofibrils, a process in which not UPS but other proteases (such as calpains, caspase 3 or cathepsin) probably play a key role (Costelli et al., 2005). Instead few E3 ligase enzymes seem to be transcriptionally induced to regulate the atrophy. The first to be identified were Atrogin-1/MAFbx and MuRF1 (De Palma et al., 2008), two E3 ligases specifically expressed in striated and smooth muscle. The involvement of Atrogin-1 and MuRF1 in atrophy has been reinforced by experiments performed in knock-out mice, which were found to be resistant to muscle atrophy induced by denervation (Nury et al., 2007). Another ligase relevant in muscle wasting is CHIP (Arndt et al., 2010), which regulates ubiquitination and the lysosomal-dependent degradation of filamin C, a muscle protein found in the Z line, and of unfolded proteins bound to Hsp70 or Hsp90. The CHIP ubiquitin ligase markedly increases in the aged muscle, reflecting adaptations of muscle during aging to eliminate more efficiently misfolded proteins (Altun et al., 2010).

Sarcopenia differs from acute disuse atrophy in several aspects: in disuse atrophy muscle mass presents a reduction in fibers number but the specific force is maintained concomitantly with fast type fibers, while in the age-related sarcopenia fibers number and specific force are all reduced and there is a shift towards the expression of slow type fibers (Lang et al., 2010). Additionally, while sarcopenia develops over months to years depending on the species considered, the rapid loss of muscle weight (20-50% loss) induced by fasting, disuse and during various catabolic diseases, can occur in rodents in several days. As far as it concerns the etiology of sarcopenia, several factors are involved, including denervation, inactivity, hormonal and nutritional changes. Further, the chronic inflammatory status which characterizes elderly subjects (inflammaging) (Franceschi et al., 2000, 2007; Cevenini et al., 2013), plays an important role also in age-related muscle weakness. Indeed, epidemiological studies in humans have shown that there is a correlation between high levels of inflammatory cytokines, low levels of IGF1, high levels of oxidative stress, decreased mitochondrial function and muscle weakness. In particular, in aged muscle an increase in TNF-α, which is one of the major inducer of i-proteasome expression, was described (Kandarian et al., 2006).

The most of studies on intracellular protein degradation by UPS in muscle wasting and sarcopenia are focused on enzymes of ubiquitination, and little is known on the roles of proteasomes, especially in humans.

Dahlmann and colleagues studied proteasomes composition in rat skeletal muscle. Approximately 95% of the proteasomes population contained the β constitutive subunits (subtype I, II) or composed by an intermediate subtypes, containing a mixture of constitutive and inducible subunits (subtypes III, IV). The immunoproteasome subtype, containing only the inducible subunits, represented a minor fraction (5%) of the total proteasomes (Dahlmann et al., 2001).

Ferrington and co-workers investigated proteasome function and subunits composition in type I fibers of aged rat soleus muscle and they found a 3-4 fold increase in content of the 20S catalytic core in aged muscle compared to young, corresponding to a 60% decrease of specific activity. Additionally, an age-related increase of β1i subunit and a significant reduction in the amount of PA28 and PA700 proteasome regulators was described (Husom et al., 2004). The same group extended these investigations by studying proteasome-enriched preparations and purified 20S proteasomes from fast-twitch skeletal muscle (type II fibers) in young and aged rats, confirming these results (Ferrington et al., 2005). A possible explanation for the lower content of regulatory complexes associated to the 20S core could be the increased demand for degradation of oxidized proteins, which are mainly degraded by 20S proteasomes.

In contrast to these findings, Altun and co-workers showed that gastrocnemius muscle wasting in aged sarcopenic rats is associated with enhanced activity of the UPS. An increase in the protein content of proteasomes subunits, but not in their corresponding mRNA, was also observed, suggesting an enhanced translation or decreased rates of proteasomes degradation (Altun et al. 2010).

To our knowledge, only one study has been performed in humans by analyzing proteasome activities in subjects affected by benign abdominal disease. No age-related modification was observed (Bossella et al., 2008).

2. AIM OF THE STUDY

As discussed in the Introduction, aging is a complex phenomenon that affects organs and tissues at a different rate. With advancing age, the skeletal muscle undergoes a progressive loss of mass and strength, a process known as sarcopenia that eventually leads to a decreased mobility and increased risk of falls and invalidity. On the other side, another organ such as the liver that is endowed with a peculiar regenerative capacity seems to be only marginally affected by aging (Schmucker et al., 2011). Accordingly, clinical data indicate that liver transplantation from aged subjects has, in specific conditions, function and duration comparable to those achievable with grafts of liver from young donors (Cescon et al., 2003; Gastaca et al., 2005; Cescon et al., 2008; Schmeding et al., 2010). The molecular mechanisms involved in these peculiar aging patterns are still largely unknown, but it is conceivable that protein degradation machineries might play an important role, as they are responsible for the maintenance of cellular homeostasis (Chondrogianni et al., 2010; Koga et al., 2011). Indeed, it has been suggested that alteration of proteostasis may contribute to the onset and progression of several age-related pathological conditions, including skeletal muscle wasting and sarcopenia (Ciechanover, 2013), as well as to the aging phenotypes (López-Otín et al., 2013).

The ubiquitin-proteasome system (UPS) is one of the most important cellular pathways for intracellular degradation of short-lived as well as damaged proteins. To date, studies on the age-related modifications of proteasomes in liver and skeletal muscle were performed prevalently in rodents, with controversial results, while only preliminary observations have been obtained in human liver (Vasuri et al., 2010) and skeletal muscle (Bossola et al., 2008). In this scenario, **the aim of the present study was to investigate and characterize in humans the age-related modifications of proteasomes of these two different organs**. To do this, we took advantage of a unique biobank of samples of both organs, collected in the framework of two larger research projects (MiUR PRIN 2008 “Pretransplant liver biological age and age-mismatch between donor and recipient as new predictors of transplant outcome” and EU FP7 MYOAGE ‘’Understanding and Combating Age-related Muscle weakness’’). The two projects provided us respectively: i) biopsies of liver used for transplantation from donors of different age; ii) biopsies of *v. lateralis* skeletal muscle and blood serum from young and old healthy volunteers as well as patients with lower limb mobility impairment (LLMI).

Proteasomes subunits expression and composition were assessed by qPCR and immunoblotting. The subcellular distribution of different proteasomes subunits was analyzed by immunohistochemistry. Proteasomes activities were assessed by fluorogenic peptides assay and indirectly, by measuring the levels of oxidized and polyubitinylated proteins. Finally, we measured c-proteasomes levels in sera of muscle donors by ELISA immune-assay and we performed correlation analyses with different anthropometric and biochemical parameters collected in the framework of the MYOAGE project.

3. MATERIALS AND METHODS

3.1 REAGENTS

Suc-Leu-Leu-Val-Tyr-MCA (3120-v), Z-Leu-Leu-Glu-MCA (3179-v) and free MCA (3099-v) fluorigenic substrates were from Peptide Institute INC (Japan), Bz-Val-Gly-Arg-MCA (BW 9375) was from Biomol (Enzo Life Sciences Inc, Farmingdale, New York, US). Proteasome inhibitor epoxomicyn (E3652) was purchased from Sigma (Saint Louis, Missouri, US). Synergy™ fluorimeter (Bio-Tek Instruments, Winooski, Vermount, US) was used to detect MCA fluorescence.

Mouse monoclonal and rabbit polyclonal anti-α4 (PW8120, clone MCP34), anti-α6 (PW8100, clone MCP20), anti-β1 (PW8140, clone MCP421), anti-β2 (PW9300, clone MCP165), anti-β5 (PW8895), anti-β1i (PW8840, clone LMP2-13), anti-β2i (PW8200), anti-β5i (PW8845, clone LMP7-1), anti-PA28α (PW8185), anti-Rpt2 (PW8305) proteasome subunits as well as the antibody against mono and poly-ubiquitin conjugates (PW8810, clone FK2) were from Biomol (Enzo Life Sciences Inc, Farmingdale, New York, USA); anti-actin (sc-8432) was from Santa Cruz Biotechnology Inc (Dallas, Texas, US) and anti-PA28β (2409) was from Cell Signaling Technology (Boston, Massachusetts, US). Oxyblot™ protein oxidation detection kit (S7150) for analysis of oxidized proteins was from Chemicon International from Pierce (#78833; Thermo Fisher Scientific, Walthman, MA). All the apparatus and reagents for SDS-PAGE and western blotting (WB), anti-mouse and -rabbit horseradish peroxidise-conjugated secondary antibodies and the QuantityOne software were from Bio-Rad (Hercules, CA, US). ECL reagent (sc-2048, Santa Cruz Biotechnology Inc), Hyperfilm ECL sheets (GEH28906837, GE Healthcare, Chalfont St. Giles, UK) and Development and Fixer processing (Kodak P7042 and P7167, Sigma) were used for the detection of immunoblot staining.

For immunohistochemistry, EnVision Plus System HRP Labelled polymer (anti-mouse, K4003; anti-rabbit K4001) and 3',3' Diaminobenzidine Substrate Chromogen System (K3468) from Dako, (Glostrup, Denmark) were used.

RNeasy mini-kit (74104, QIAGEN, Germany) was used to extract RNA from skeletal muscle cell pellets, while miRNA isolation kit (AM1560, Ambion, US) was used for the extraction of total RNA from skeletal muscle tissue. UV light transilluminator (Gel Doc 2000, Bio-Rad) was used to

analyze extracted RNA. SuperScript III Reverse Transcriptase (18080, Invitrogen, US), random primers and dNTPs were used for cDNA synthesis from total RNA of skeletal muscle tissue. MESA Blue qPCR MasterMix Plus for SYBR® Assay No ROXsolution (RT-SY2X-06+NRWOU,Eurogentec, Belgium) was used for the quantitative expression analysis of proteasome subunits in skeletal muscle tissue.

Primers were designed on the genomic sequence present in the Genbank database (National Center for Biotechnology Information, NCBI) and by using the Primer-BLAST tool. Instrument Rotor Gene-Q (R0610137, QIAGEN, Hilden Germany) was used for the quantitative analysis of mRNA of proteasome subunits.

Immunoplate Nunc Maxisorp (YO-01928-08, Thermoscientific, US), anti-α6 (PW8100, Enzo Lifesciences), rabbit polyclonal Ab clone K42 (produced at the Institute of Biochemistry Charité, Berlin Germany), peroxidase-conjugated goat anti-rabbit IgG (111-035-003, Jackson ImmunoResearch, West Grove, Chester County, PA, US), tetramethylbenzidine (T0440, Sigma-Aldrich, US) were used to perform ELISA assay. All the other solutions were home made.

3.2 HUMAN SAMPLE BIOBANK

3.2.1 STUDY OF HUMAN LIVER

Human liver specimens from organ used for transplantation were collected in the context of the PRIN08 project "Pretransplant liver biological age and age-mismatch between donor and recipient as new predictors of transplant outcome" with the collaboration of Professor Gian Luca Grazi .The study was approved by the local Ethical Committee at S. Orsola-Malpighi Hospital (2008, June 26th code: 44/2008/O/Tess). Liver biopsies were obtained in the framework of the protocol of donor-recipient allogenic liver graft performed at General Surgery and Transplant Unit (S. Orsola-Malpighi Hospital, Bologna, Italy) between 2008 and 2010 from heart-beating donors before engraftment. Fifty-six samples were used in the present studies; age and gender distribution of the donors are summarised in **table 1**.

Liver specimens of donors			
Age group	**1**: ≤40 years	**2**: 41-69 years	**3**: ≥70 years
Number of samples	9	31	16
Donor gender	0F-9M	17F-14M	6F-10M
Mean age±SD	24.1±6.3	56.5±9.1	77.6±4.7

Table 1. Age and gender distribution of the liver biopsies collected in the context of donor-recipient allogenic graft and analyzed in the study. M=male donors; F=female donors; SD=standard deviation.

Pre-transplant liver biopsy was performed in young donors with some alterations in serum liver function test or in presence of obesity and other metabolic diseases, as well as virtually in all elderly donors (≥70 years).

The biopsies were subdivided according to the age of the donor in three groups (age group 1: ≤40 years; age group 2: 41-70 years; age group 3: ≥70 years) on the basis of previous literature (Vo et al. 2011).

3.2.2 STUDY OF HUMAN SKELETAL MUSCLE

The samples analyzed were collected within the framework of the FP7 European project ''MYOAGE-Understanding and Combating Age-related Muscle weakness'', Coordinator Prof. Gillian Butler-Browne we were involved in.

Two different groups of subjects were recruited: patients with limited lower limb mobility impairment (**LLMI patients**) and **healthy volunteers** (McPhee et al., 2013). The study was approved by the local ethic committees of the operative units (as far as the Bologna Unit: Rizzoli Hospital ethical committee, 2010, April 26, code 10823, class 07-03) and written informed consent for study procedures was obtained from patients and volunteers.

LLMI patients undergoing surgery for hip replacement at Rizzoli Hospital in Bologna, were included. Patients groups were matched for biopsy location and chronological age. Exclusion criteria were: chronic kidney diseases or liver pathologies, bleeding disorders, severe diabetes mellitus, rheumatic disease other than osteoarthritis, neuromuscular disorders, malignancies, systemic infections, chronic steroid use, psychological problems, history of alcohol or drug abuse, evidence of prior surgery that involves the hip.

Additionally, biopsies of human *vastus lateralis* (*v. lateralis*) muscle were collected during surgery (2 specimens of 450 mg for each subject), frozen in liquid nitrogen and stored at -80°C.

Numbers and distribution of LLMI patients studied are reported in **table 2**.

Muscle specimens of LLMI patients			
Age group	≤40 years	41-70 years	≥70 years
Number of samples	15	26	16
Donor gender	6F-9M	12F-14M	10F-6M
Mean age±SD	32.8 ± 4.6	55.6 ± 8.1	85.2 ± 8

Table 2. Bioptic specimens of human *vastus lateralis* muscle used for molecular investigations. M=male donors; F=female donors; SD=standard deviation.

The **healthy group**, was composed by young (≤40 years), and cognitively active elderly volunteers (≥70 years) without clinical signs of muscle weakness, recruited in different European centers, including the University of Leiden (The Netherlands), whose samples were analyzed in the present study. The subjects considered eligible for the study were found to be healthy on the basis of different parameters, such as clinical history, including a standard questionnaire on the activities of daily living, physical examination and screening tests. Exclusion criteria, in order to select healthy participants and minimize the confounding effect of diseases on muscle mass, were as follows: living situation dependent on others, inability to walk a distance of 250m, presence of comorbidity (neurological disorders, metabolic and rheumatic diseases), coagulation syndromes, use of specific drugs (immunosuppressive drugs, insulin, anticoagulation), immobilization for one

week during last three months and orthopedic surgery during the last two years or still cause of functional limitations. Subjects young and old involved in competitive sports and master athletes were also excluded.

Small biopsies from *v. lateralis* skeletal muscle (150 mg for subject) were obtained with modified Bergstrom needle and were immediately frozen in liquid nitrogen and stored at -80°C (**table 3**).

Muscle specimens of healthy subjects		
Age group	≤40 years	≥70 years
Number of samples	5	9
Donor gender	5F	9F
Mean age±SD	21.4 ± 4.3	75.6 ± 2.7

Table 3. Muscle specimens of healthy subjects used for molecular investigations. M=male donors; F=female donors; SD=standard deviation.

Additionally, satellite cells were isolated from muscle biopsies of young and elderly healthy subjects and cultured *in vitro*, at different oxygen tension (5% and 21%) at the University of Namur. Frozen pellets of myoblasts were provided for molecular investigations (**table 4**).

Myoblasts of healthy subjects		
Age group	≤40 years	≥70 years
Number of samples	3	5

Table 4. Myoblast cell cultures analyzed.

Finally, peripheral blood samples (40 ml for each subject) from LLMI patients (**table 5**) and healthy subjects (**table 6**) were collected before surgery and then processed in order to obtain: plasma, serum, peripheral blood mononuclear cells and granulocytes.

LLMI patients serum			
Age group	≤40 years	41-69 years	≥70 years
Number of samples	11	15	20
Donor gender	5F-6M	5F-10M	14F-6M
Mean age±SD	33.7 ± 5.2	55.8 ± 8.3	84.2 ± 7.7

Table 5. Serum of LLMI patients collected and analyzed. M=male donors; F=female donors; SD=standard deviation.

Healthy subjects serum		
Age group	≤40 years	≥70 years
Number of samples	33	67
Donor gender	19F-14M	31F-36M
Mean age±SD	21.6 ± 2.8	74.1 ± 3

Table 6. Serum from healthy subjects collected at University of Leiden and analyzed in this study. M=male donors; F=female donors; SD=standard deviation.

3.3 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Liver biopsies were collected in 54 cases for routine evaluation of graft suitability for transplantation on frozen sections, following the pre-transplant protocol for transplant safety (Fiorentino M, et al. 2009). In 2 cases no liver biopsy was performed. After evaluation, biopsies were formalin fixed, paraffin embedded and routinely processed. Slices of two-µm-thick were cut and stained with Haematoxylin-Eosin. The following histological parameters were collected:

occurrence of portal inflammation and/or lobular inflammation, fibrosis (according to Ishak et al., 1995), arteriolar myointimal thickening, biliocytes regression, occurrence and amount of cholestasis, polymorphism of hepatocytes, sinusoid dilatation, percentage of steatosis (both micro- and macro-vesicular), lipofuscin accumulation.

Immunohistochemistry (IHC) for specific antibodies *versus* β1, β1i, β5i, and PA28α proteasomes subunits, was performed in 54 cases as previously described (Mishto et al., 2011). The nuclear and cytoplasmic intensity of the signal to the antibody was assessed semi-quantitatively in 0 (absent), 1+ (mild/average) and 2+ (strong), as previously described (Vasuri et al., 2010).

Biopsies of skeletal muscle were processed with the same experimental protocol above described. The obtained sections were analyzed for specific antibodies versus β1, β1i, β5i proteasomes subunits.

3.4 PREPARATION OF PROTEIN CRUDE EXTRACTS

Protein crude extracts were prepared as previously described (Schmidt et al., 2006; Vasuri et al., 2010). An amount of 50 milligrams of liver frozen tissue was lysed in TEAD buffer (Tris-HCl 20 mM pH=7.5, EDTA 1mM, NaN_3 1mM, DTT 1mM), homogenized using a motor-driven homogenizer and centrifuged at 25000 g for 1h at 4°C. The supernatant containing the soluble proteins (crude extract) was collected, quantified by Bradford's method and stored at -80°C.

The same protocol was used to prepare crude protein extracts from about 70-100 mg of frozen skeletal muscle biopsies, (Dahlmann et al., 2001) and from pellets of about $3*10^6$ myoblast cells.

3.5 20S PROTEASOME PURIFICATION

Purification of 20S liver proteasomes was performed similar as described elsewhere (Schmidt et al., 2006) at the Institute of Biochemistry, Charitè, Berlin. Briefly, two pools of liver specimens from adult (n=4; mean age 48±4.5 years) and old (n=3; mean age 85±3.5 years) male

donors were homogenized in TEAD buffer, as above described and the not dissolved material pelleted by centrifugation for 20 minutes at 15000 g and 4°C. Proteins in the supernatant fraction were loaded onto a DEAE-Toyopearl column equilibrated with TEAD buffer and bound proteins then eluted with a linear gradient of 0-0.5 M NaCl/TEAD buffer. Proteasome containing fractions were pooled and concentrated by adding $(NH_4)_2SO_4$ to obtain a final concentration of 75% (w/v) respect to the salt. Proteins were spun down and then dissolved in TEAD buffer before loaded onto a Superose 6 gel filtration column for chromatography in TEAD buffer in a FPLC System (GE Healthcare). Fractions containing 20S proteasome were pooled and further purified by subsequent chromatography on Mono Q and Phenyl-Superose. Finally, proteasomes were dialyzed against TEAD buffer.

3.6 PROTEASOME ACTIVITY ASSAY

Proteasome activities were measured in crude protein extracts of liver and LLMI patients skeletal muscle and in purified 20S liver proteasome by using the fluorogenic substrates Suc-LLVY-MCA, Bz-VGR-MCA and Z-LLE-MCA, specific for chymotryptic-, tryptic-, and caspase-like activities, as already described (Dahlmann et al., 2000; Mishto et al. 2006a). An amount of 10 µg of crude protein extracts of liver proteins, 20 µg of skeletal muscle proteins respectively and 0,1 µg of purified 20S proteasome were incubated in presence of 200µM of Suc-LLVY-MCA and Z-LLE-MCA and 600 µM of Bz-VGR-MCA in TEAD buffer, at 37°C for 1h, in a 96-well plate. The enzymatically released MCA was measured by fluorometer at 360 nm excitation and 460 nm emission. Proteasome activity was expressed as specific activity (nmol of free MCA/min for mg protein). The specificity of the reaction was assessed measuring the fluorescence released in presence of the proteasome inhibitor epoxomycin (10 µM).

3.7 WESTERN BLOTTING ANALYSIS

Western blotting analysis of protein crude extracts from liver and skeletal muscle as well as purified 20S liver proteasomes was performed as previously described (Mishto et al., 2006a;

Bellavista et al., 2008). An amount of 40 µg of crude extracts or 0.5 µg of 20S purified proteasomes were separated in a 16% polyacrylamide gel and were transferred to a nitrocellulose filter. The proteasome subunits were detected using primary antibodies above described, overnight incubation at 4°C, shaking in TBS solution with 5% of non-fat dry milk and 0.01% of Tween 20. Densitometry analysis (INT*mm2) of the band corresponding to each subunit was performed using QuantityOne Software. Each experiment was repeated and we considered the mean of results. In order to compare samples loaded in different gels, a liver and a skeletal muscle samples were used as internal control and loaded on each gel of liver and muscle sets respectively. Data are reported as relative content between the samples and the control's band density. Protein isoloading was assessed by SDS-PAGE and Coomassie dye staining.

3.8 OXIDIZED PROTEINS AND POLY-UBIQUITIN CONJUGATES DETECTION

The content of carbonyl groups, representative of oxidative protein modification, was determined in 15 µg of protein crude extracts from liver and LLMI patient skeletal muscle obtained from donors of different age by using the Oxyblot^{TM} protein oxidation detection kit according to manufacturer's instruction, SDS-PAGE and immunoblotting analysis.

Levels of poly-ubiquitin conjugated proteins were measured in 20 µg of protein crude extracts of the above described samples by SDS-PAGE and immunoblotting analysis coupled to the FK2 monoclonal antibody, specific for K^{29}-, K^{48}-, and K^{63}- linked conjugates.

3.9 2D-PAGE OF 20S PROTEASOMES

Purified 20S proteasomes from liver biopsies were precipitated with an equal volume of ethanol and then subjected to two-dimensional non-equilibrium pH polyacrylamide gel electrophoresis as previously described (Mishto et al. 2006b).

3.10 REAL-TIME POLYMERASE CHAIN REACTION

To analyze the expression of a subset of target genes coding for proteasomes subunits, in skeletal muscle biopsies, we adopted the qPCR technique. We extracted RNA from C520 and T2 cell lines as positive and negative controls for immunoproteasome respectively. C520 is a lymphoblastoid cell line obtained from EBV-immortalyzed B lymphocytes, expressing predominantly immunoproteasome, whereas the T2 cell line is a T-B lymphoblast hybrid (ATCC CRL-1992) carrying a deletion in the region of chromosome 6 encoding for β1i, β5i, TAP1, TAP2 genes and therefore this cell line expresses only the constitutive proteasome.

3.10.1 RNA EXTRACTION FROM CELL LINES

We extracted RNA from cell pellets by using RNeasy mini-kit (QIAGEN). According to the protocol including in the kit, we firstly added 600ul of RTL lysis buffer for an amount of cells of $5*10^6$-$1*10^7$. The obtained RNA was stored at -80°C. The quality of products was assessed by electrophoresis analysis in a 1% agarose gel, to verify the characteristic pattern of rRNAs.

3.10.2 RNA EXTRACTION FROM MUSCLE BIOPSIES

The extraction of total RNA from biopsies of skeletal muscle tissue was performed using the miRNA isolation kit (Ambion), according to the manufacture indications reported in the same kit. Approximately 40 mg of each biopsy was lysed by adding a lysis buffer in a volume equal to 10 times the weight of the sample and by using a motor-driven homogenizer. Products of extraction were treated with DNase enzyme to remove all DNA, that might interfere with subsequent analysis. The RNA was stored at -80°C.

3.10.3 RNA QUANTIFICATION

RNA integrity was assessed by electrophoresis analysis in a 1% agarose gel and by using the UV light transilluminator (Bio-Rad). We evaluated the presence of bands corresponding to ribosomal RNA: 28S, 18S and 5S. The RNA was analyzed spectrophotometrically to determine its

concentration and to highlight possible contamination by phenol or protein. The proteins, which are the main contaminants of the extracts, are measured directly at 280 nm, while nucleic acids at 260 nm. The A260/A280 ratio was used to estimate the purity of the RNA, typically in pure preparations of nucleic acids this ratio is 1.8 for DNA and 2.0 for RNA. Instead, the absorbance at 230nm indicates the contamination of the sample due to substances such as carbohydrates, phenols, aromatic compounds or peptides. In the case of pure samples the A260/A230 ratio should be approximately 2.2. Preparations in which the mentioned relationships differ significantly from those of pure preparations are index of contamination and this makes the estimation of the concentration of obtained RNA less accurate.

3.10.4 cDNA SYNTHESIS

For cDNA synthesis, 750 ng of total RNA were inversely transcribed using the SuperScript III Reverse Transcriptase (Invitrogen). For each RNA sample we added 0.5 µl of random primers (3 µg/µL), 1µl of dNTPs (10 mM each) and sterile water up to 12.5 µl. The mixture was incubated for 5' at 65°C to denature the RNA and activate the primers. Subsequently, we added 4 µl of the First-Strand Buffer 5X, 1 µl of DTT 0,1 M, 0.5 µl of the SuperScript Reverse Transcriptase (200 units/µl) and sterile water to reach 20 µl of volume. The thermal profile of synthesis was: 25°C-5', 50°C-50', 72°C-15', 4°C. The obtained cDNA was diluted 1:10 and stored at -80°C.

3.10.5 qPCR

Quantitative analysis of proteasome subunits was performed by Real-time Polymerase Chain Reaction (PCR quantitative relative analysis). The gene of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a housekeeping, to normalize the levels of starting cDNA. The reaction was performed using the MESA Blue qPCR MasterMix Plus for SYBR® Assay No ROXsolution (Eurogentec) according to the protocol of the company, in association with specific primers below described (**table 7**).

Primer	Length	T_m	Sequence 5'→3'
Gapdh For	24	60°C	CATTGCCCTCAACGACCACTTTGT
Gapdh Rev	26	60 °C	TCTCTCTCTTCCTCTTGTGCTCTTGC
α6 For	20	53°C	AATTCTCCATGTTGACAACC
α6 Rev	20	55°C	AGATACAAGACGAGACACAG
β1 For	20	59°C	AGACTGGGAAAGCCGAGAAG
β1 Rev	20	57°C	GCAGAAAATGCGGTCGTGAA
Lmp2 For	22	60°C	CGTTGTGATGGGTTCTGATTCC
Lmp2 Rev	23	61°C	GACAGCTTGTCAAACACTCGGTT
Lmp7 For	18	61°C	GCAGTGGATTCTCGGGCC
Lmp7 Rev	24	63°C	GCCAAGCAGGTAAGGGTTAATCTC
Pa28β For	15	59°C	GTGCGCCTGAGCGGG
Pa28β Rev	23	61°C	TCAGCCTCCTGGAAAAGATTCTG
α4 For	20	65°C	CCGCCGGCATGAGCTACGAC
α4 Rev	21	66°C	CGCGGTCGAGCCCTTCTTGAC

Table 7. Primers used in qPCR.

The amplification conditions used in the standard PCR reaction were the following: a step of pre-incubation at 95°C for 3', followed by 35 cycles of denaturation at 93°C for 15", annealing step at 60°C for 20" and an extension step at 72°C for 1'. The quality of products was verified by electrophoresis analysis in a 2% agarose gel.

Once assessed the specificity of primers, the amplification of transcripts was performed with the instrument Rotor Gene-Q (QIAGEN) under the following conditions: 5 µl of Master Mix 2X, 0.15 µl of Forward Primer (10 µM), 0.15 µl of Reverse Primer (10 µM), 1 µl of cDNA (diluted 1:10), 3.7 µl of H_2O milli-Q.

The thermal profile was: 95°C for 5', followed by 45 cycles at 95°C for 15", 60°C for 1' and 72°C for 20". At the end of the polymerase reaction, a melting cycle of progressive denaturation was performed to confirm the presence of specific amplicons in the reaction. All samples were

amplified in duplicate; negative controls, without the template cDNA, were used in each assay. A sample was used as internal calibrator in each run. The analysis of the results was performed according to the comparative method with evaluation of the threshold cycle (Ct). The ΔCt value was calculated by subtracting the Ct value of housekeeping gene from the Ct value of the target gene, for a sample used as calibrator and for all samples. The ΔΔCt value was then calculated by subtracting the ΔCt value of calibrator from the ΔCt of each sample. In the ΔΔCt method to obtain a correct quantification, the efficiencies of PCR of the target gene and the reference gene should be close to 2 (maximum efficiency) or 1.8 (good efficiency). The relative expression level was then determined by calculating this expression [$1.8(^{-\Delta\Delta Ct})$], which indicates how many times, more or less, the gene of interest is expressed in the analyzed sample compared to the calibrator (Pfaffl, 2001).

3.11 ELISA ASSAY

To detect circulating proteasomes (c-proteasomes) in serum of LLMI patients and healthy subjects we adopted an Enzyme-Linked Immunosorbent Assay (ELISA) (Mueller et al., 2012). In particular, we performed in our laboratory an heterogeneous, direct and non-competitive ELISA assay as below described.

3.11.1 COATING

In the first step, the antibody specific for the analyte of interest was linked to the bottom of a 96 well plate, the plate Nunc Maxisorp (Thermoscientific), in order to create a solid phase of capture. We used a monoclonal antibody *versus* α6 protesomes subunit (Enzo Lifesciences) diluted 1:1500 in sterile PBS at pH 7.4 (100ul/well), incubated overnight at 4°C, no shaking.

3.11.2 BLOCKING

In this step, we washed the plate 5 times with 300 µL of PBS-T 0.1% and we added 300 µL of PBS-T 0.1%-BSA 1% (blocking buffer) to each well to prevent non-specific bindings of the

analyte. Then we incubated the plate for 6 h in shaking at room temperature and subsequently washed the plate as above described.

3.11.3 SAMPLES AND STANDARD CURVE

To determine the concentration of c-proteasomes in sera it was necessary to set up a standard curve with 20S purified proteasomes. We used 20S proteasome, purified from human lymphoblastoid cell lines and concentrated 77 ug/ml. We prepared serial dilutions of the 20S proteasome from 0 to 100 ng/ml (0, 6.25, 12.5, 25, 50, 100 ng/ml) in PBS-T 0.1%-BSA 1% (100 µl/well) in triplicate. Serum samples were diluted 1:15 and/or 1:30 in PBS-T 0.1%-BSA 1% (100 µl/well) in triplicate. In order to normalize the experimental variability, we loaded serum of an healthy subject as internal control in each plate. The plate with the standard curve and samples was incubated overnight at 4°C, in shaking.

3.11.4 INCUBATION WITH ANTI-CORE PROTEASOME ANTIBODY

The following day we washed the plate 3 times as above described and we added polyclonal anti-core proteasomes antibody (K42 clone, Charité 's laboratory stock), that binds , α5, α7, β7, and β5i proteasomes subunits. We used this antibody diluted 1:750 in PBS-T 0.1% (100 µl/well) and we incubated the plate for 3h at 4°C, in shaking.

3.11.5 INCUBATION WITH SECONDARY ANTIBODY

In the last step we washed the plate 5 times as above described and we added the peroxidase-conjugated anti-rabbit IgG secondary antibody for the detection of the antigen-antibody complex. The antibody was diluted 1:7500 in PBS-T 0.1% (100ul/well) and we incubated the plate 1h at 4°C, in shaking.

3.11.6 DETECTION

The detection system adopted entails the use of tetramethylbenzidine (TMB) as substrate of the peroxidase enzyme. The reaction leads to the formation of a blue colored product, whose intensity is proportional to the amount of analyte detected; subsequently the reaction has to be stopped with an acid solution, that turns the color from blue to yellow. Finally a plate reader analyzes the signal emitted at 450nm.

We washed the plate 5 times as above described, we added 100uL of TMB and we incubated the plate at room temperature, 10' at dark. Lastly the reaction was stopped with 100 µl of H_2SO_4 stopping solution and the absorbance of samples was measured.

3.11.7 DATA ANALYSIS

Taking into account that we used a non-competitive ELISA, all the analyte present in the samples is captured by the excess of antibody and revealed, thus the signal analyzed is directly proportional to the concentration of analyte in the samples and the dose-response curve is linear. By analyzing the quality of the standard curve, our calibration curve of 20S proteasome reached the saturation close to the concentration of 100 ng/ml, whereby this value was eliminated from the analysis.

We calculated the average of measures in triplicate and the analyte concentration was obtained by interpolation on the calibration curve.

$$y=ax + b$$

where x is the concentration of the target substance in the sample to be analyzed and y is the absorbance.

3.12 STATISTICAL ANALYSIS

Data were analyzed by one-way-ANOVA, Kruskal-Wallis, Student's *t* or Mann-Whitney tests to assess differences among age groups. Spearman's test was applied to assess the correlations between age histological parameters and Immunohistochemistry, as well as c-proteasomes level

with anthropometric parameters and cytokines in serum. Gender of donor was also taken into consideration for analyses on liver and skeletal muscle tissue, as well as human serum. All values are reported as mean of three independent experiments; data are presented as graphs box. Statistical comparisons were performed using STATA software version 9.0 (Stata Corp. Texas, USA) and a *P*-value<0.05 was considered as statistically significant.

4. RESULTS ON HUMAN LIVER

4.1 HISTOLOGICAL PROFILE OF AGED HUMAN LIVER

Human liver biopsies of organs used for transplantation were collected in the framework of the PRIN08 project "Pretransplant liver biological age and age-mismatch between donor and recipient as new predictors of transplant outcome", which aimed to identify biomarkers of human liver biological aging and not of chronological age in liver donors, in the context of organ transplant.

Before investigating whether aging impinges upon proteasomes in human liver, on the same biopsies we performed histopathological analysis. The main parameters taken into account were the presence of portal inflammation and lobular necrosis. Indeed, we have previously shown that, in pathological conditions such as cirrhosis and chronic active hepatitis, the levels of β1i and β5i i-subunits and of the PA28α regulatory complex correlate with the degree of tissue inflammation (Vasuri et al., 2010), thus all samples that showed signs of active inflammation, like piecemeal necrosis or lobular necrosis, with a score ≥1 in one or more parameters of the Ishak's score were excluded from analysis, in total two samples were excluded. On the contrary, cases with mild portal lymphocytic inflammation but without piecemeal necrosis, which can be observed even in the absence of a true hepatitis, were included.

The **histological analysis** of the 54 liver biopsies (2 out of 56 were not available) showed the occurrence of a mild portal chronic infiltrate in 17 (31%) cases, with no piecemeal necrosis or lobular necrosis. Fibrosis was scored as absent in 14 (26%) cases, Ishak grade 1 in 19 (35%) cases and Ishak grade 2 in 21 (39%) cases, the myointimal thickening was present in 33 (61%) cases, biliocytes regression in 15 (37%), signs of mild cholestasis in 3 (5%), sinusoid dilatation in 8 (15%) cases. Hepatocytes polymorphism was absent in 18 (33%) cases, mild in 26 (48%) and pronounced in 10 (18%) cases. A moderate-to-severe lipofuscin accumulation was observed in 20 (37%) cases. Some amount of steatosis was present in 29 (54%) cases, with a mean percentage of 5.42±10.7 (range 0-60%).

The histological parameters that directly correlated with age were fibrosis (Spearman's Test $p<0.001$), myointimal thickening ($p<0.001$), biliocytes regression ($p=0.012$), hepatocytes polymorphism ($p=0.01$) and lipofuscin accumulation ($p<0.001$) (**table 8**, **fig. 4**.) Remarkably, none

of these parameters correlated with proteasomes composition, distribution and functionality, below described.

Histological parameters		
	C	**p**
Fibrosis	0.680	<0.001
Myointimal thickening	0.626	<0.001
Biliocytes regression	0.344	0.012
Hepatocytes polymorphism	0.352	0.01
Lipofuscin accumulation	0.595	<0.001

Table 8. Correlation analysis of age with histological parameters in all liver biopsies (n=54). In Table are shown positive correlations between aging and different histological features. C=Spearman's correlation coefficient.

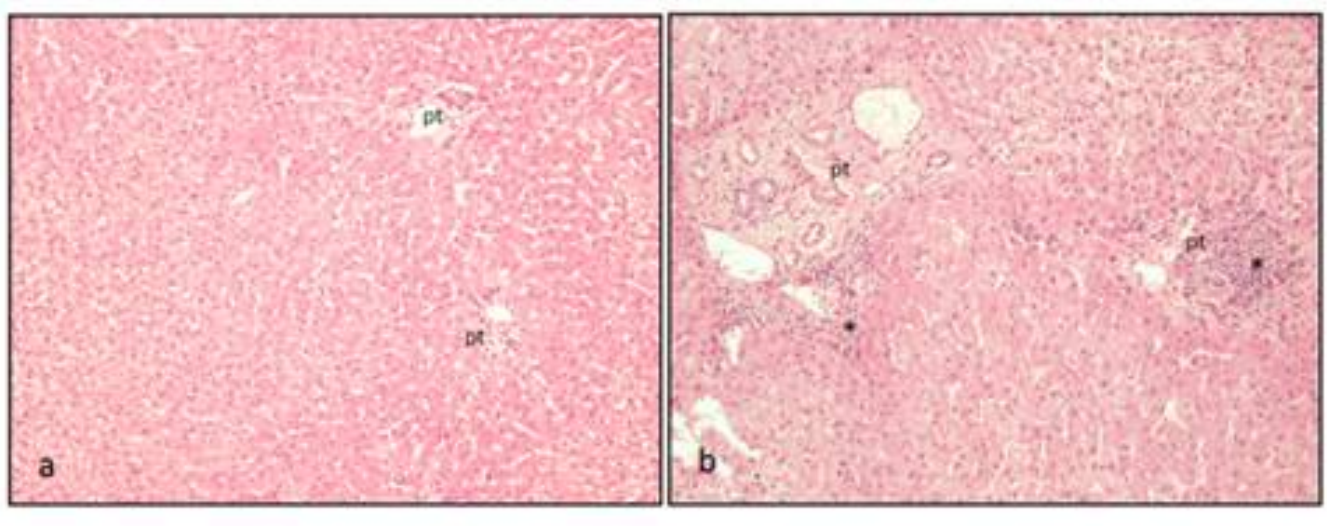


Figure 4. Histological features of livers from representative young and old donors. Note the concentric portal fibrosis, with arteriolar myointimal thickening and some amount of chronic flogistic infiltrate (asterisks) in the liver from old donor (**b**), compared to the young one (**a**). pt: portal tract. 10x magnification.

4.2 PROTEASOME COMPOSITION AND CONTENT

Starting from the results we obtained in the liver of healthy adult subjects and under pathological conditions (Vasuri et al., 2010), we firstly examined the composition and content of 20S proteasomes subunits, as well as of the PA28αβ and PA700 regulators, in the liver of different age donors.

Western blotting analysis showed that the constitutive (β1, β2, β5) and inducible (β1i, β2i, β5i) catalytic subunits, as well as PA28α and Rpt2, representative of PA28αβ and PA700 regulatory complexes respectively, were expressed in all liver biopsies (**fig. 5a, c**). Additionally, the purification of 20S proteasome from liver biopsies of adult (n=4) and elderly (n=3) male subjects and subsequent western blotting analysis, confirmed that both constitutive and inducible catalytic subunits were incorporated into mature and proteolitically active 20S proteasomes (**fig. 5b, d**). The 2D-PAGE maps also showed that most of the proteasome subunits were present in multiple isoforms, likely due to post-translational modifications (Sixt et al., 2012; Schmidt et al., 2006), in both the young and in the old group (**fig. 5e, f**).

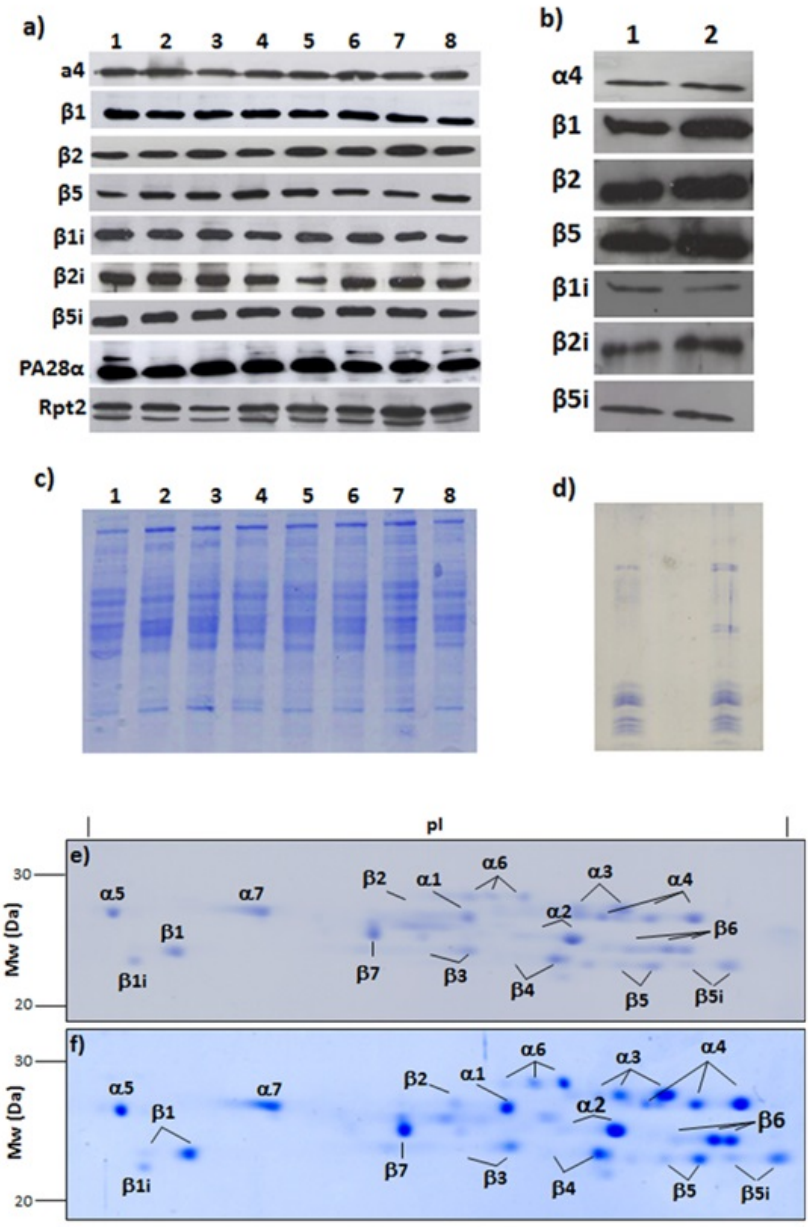
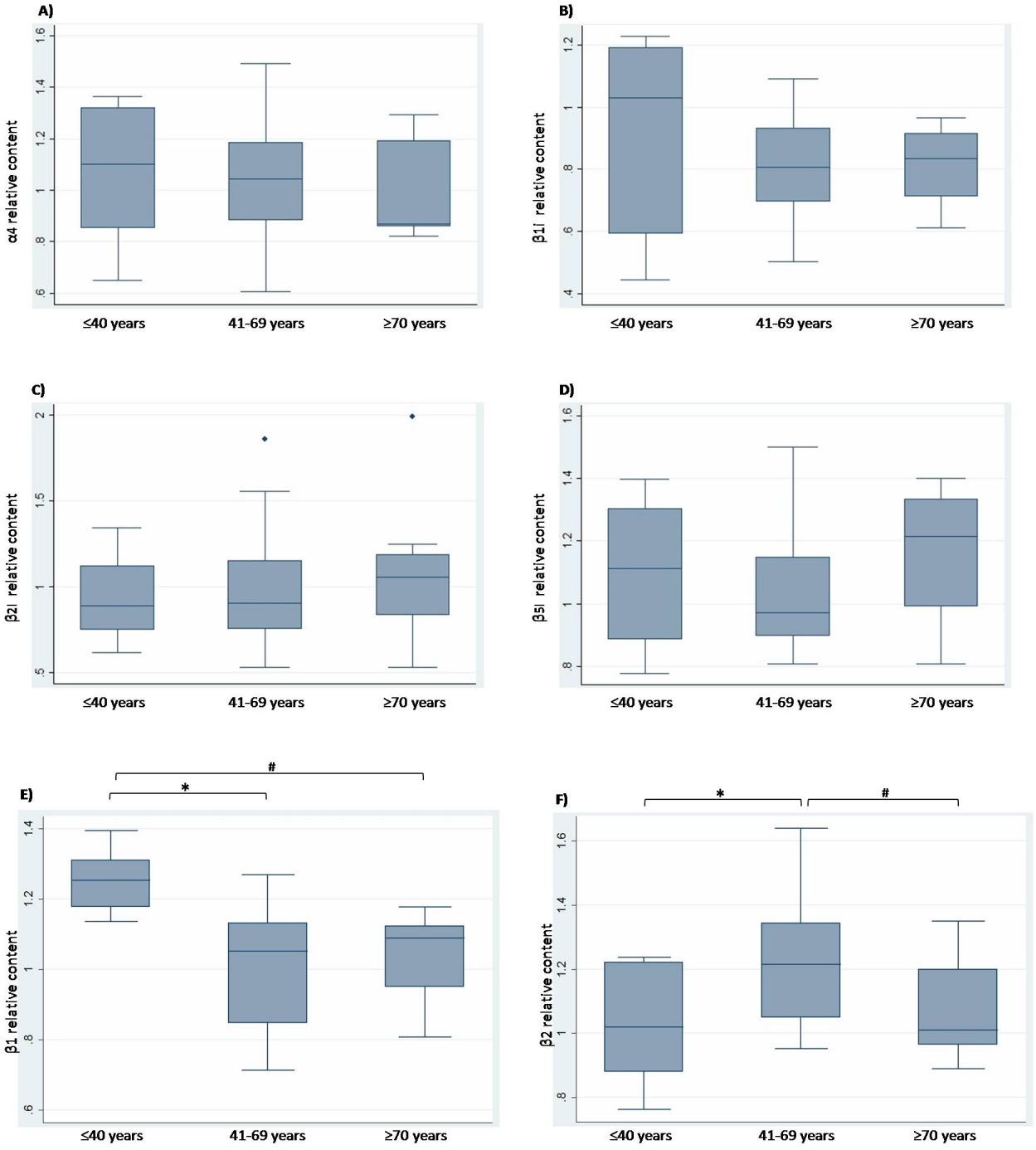


Figure 5. **Proteasomes composition of different age human livers**. Example of western blotting analysis on crude protein extracts from human liver biopsies of different age donors (1=23 years, 2=30 years, 3=43 years, 4=44 years, 5=58 years, 6=58 years, 7=70 years, 8=84 years) (**a**), and on 20S proteasomes (**b**) purified from a pool of human liver samples from adult (marked as 1; n=4, mean age 48±4.5 years) and old (marked as 2; n=3, mean age 85±3.5 years) male donors. (**c**, **d**) Coomassie gel staining of samples in picture (**a**) and (**b**) was used to assess protein isoloading. 2-D PAGE maps of 20S purified proteasomes from adult (**e**) and old (**f**) pooled liver samples as in (**b**). Different protein species of 20S proteasome subunits have been identified.

By performing semi-quantitative western blotting analyses we found no age-related variation in the expression levels of α4, β1i, β2i, β5i, β5 subunits (**fig. 6A-D, G**). On the contrary an

age-related decrease of β1 subunit (p=0.0022) (**fig. 6E**) and a non-monotonic change of β2 were observed in the whole sample size (p=0.019) (**fig. 6F**). We concomitantly analyzed the expression levels of PA28αβ and PA700 proteasome regulators, represented by PA28α and Rpt2 subunits respectively. We found that Rpt2 content was not affected by aging in human liver (**fig. 6H**) while the amount of PA28α subunit tends to decrease with age (p=0.05) (**fig. 6I**).



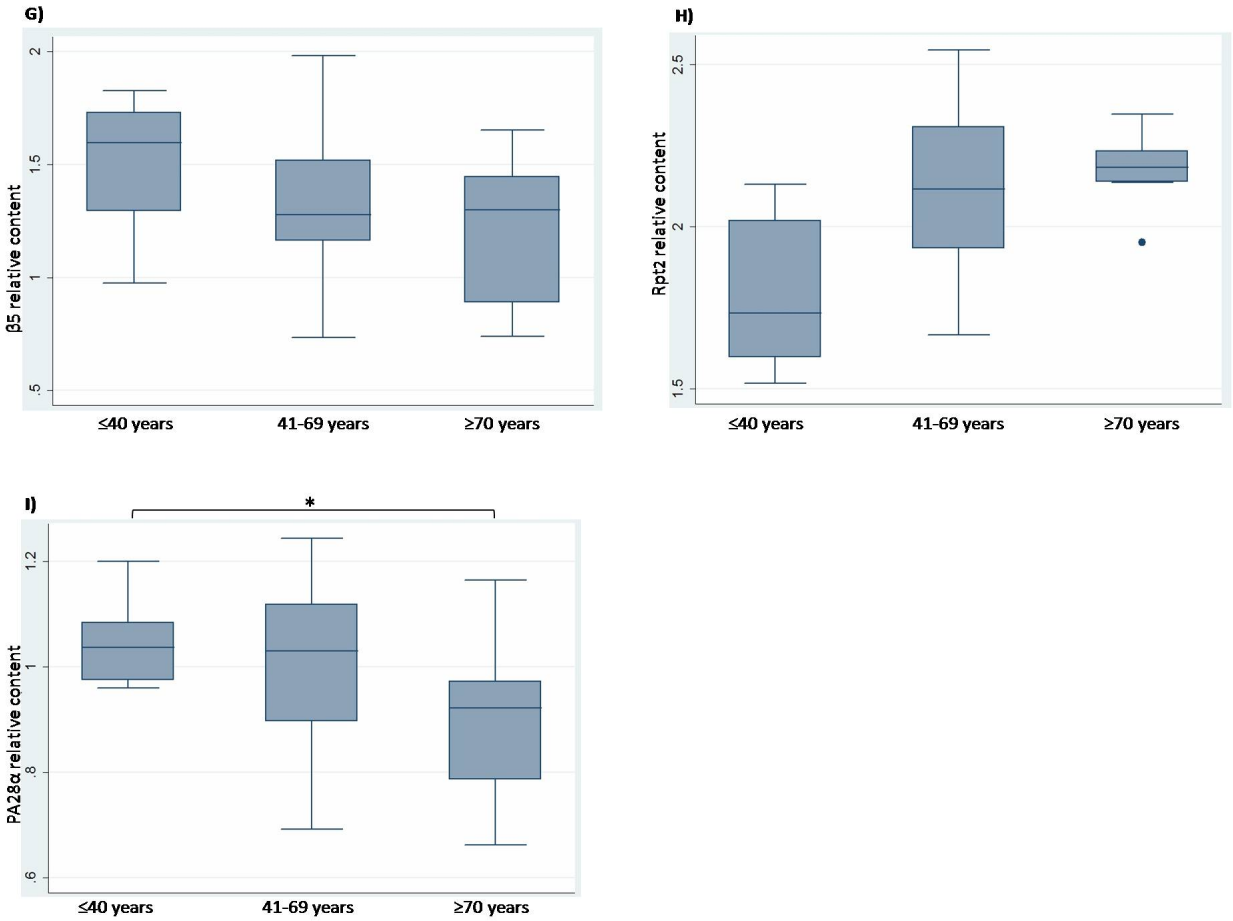
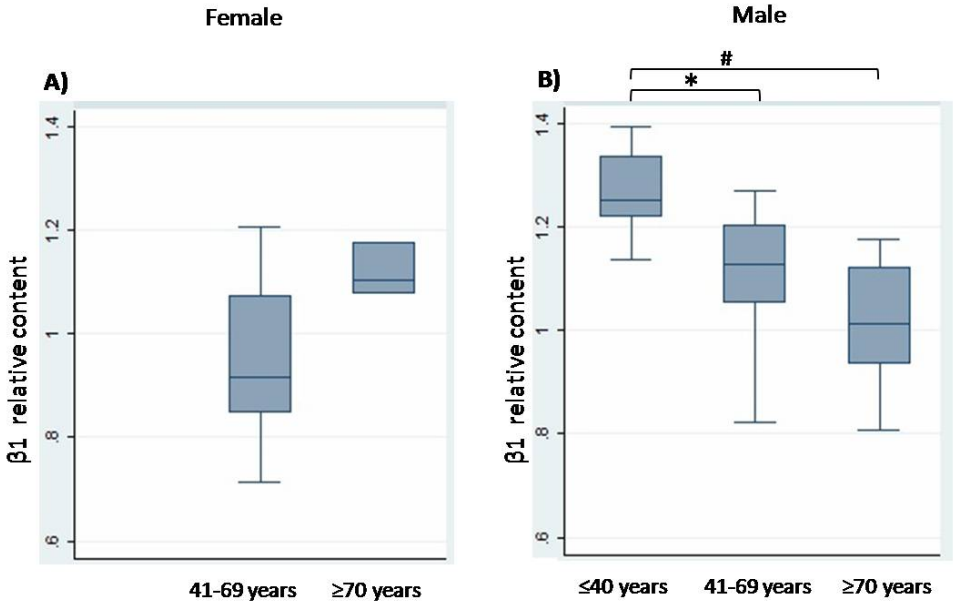
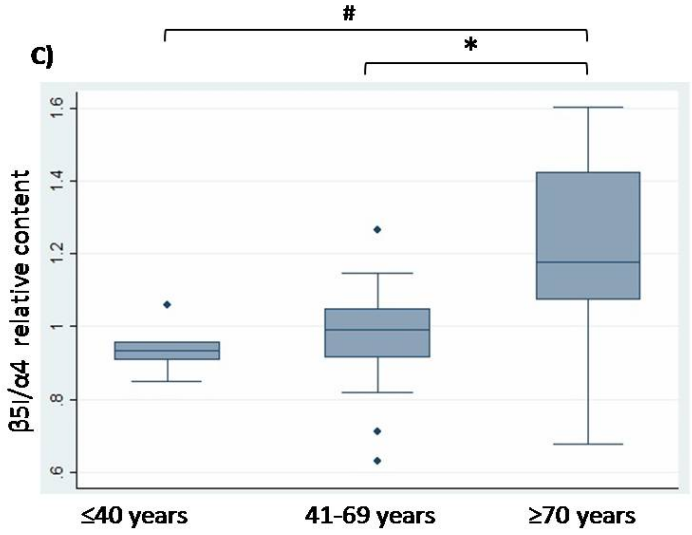


Figure 6. Relative content of proteasomes subunits in human livers of young (≤40 years), middle age (41-69 years) and elderly (≥70 years) subjects. Western blotting analysis of proteasomes subunits were performed on crude protein extracts from human livers of young (≤40 years, n=7), middle age (41-69 years, n=26) and elderly (≥70 years, n=9) subjects. Densitometric analysis of the bands corresponding to the proteasome subunits analyzed was considered and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. The relative content of α4 (**A**), β1i (**B**), β2i (**C**), β5i (**D**), β5 (**G**) proteasomes subunits in whole sample size was preserved during aging. An age-related decrease of β1 subunit was observed (one-way ANOVA p=0.0022; age group 1vs2 *p=0.0012, age group 1vs3 $^{\#}$p=0.0017, age group 2vs3 p=0.56) (**E**). A non-monotonic age-related variation of β2 was also observed (one-way ANOVA p=0.019; age group 1vs2 *p=0.02, age group 1vs3 p=0.34, age group 2vs3 $^{\#}$p=0.038) (**F**). The relative content of Rpt2 subunit of PA700 regulator was not affected by aging (**H**), while a decrease in PA28α content representative of PA28αβ regulator was observed by comparing the young and the old age group (one way ANOVA p=0.05; age group 1vs2 p=0.335, age group 1vs3 $^{\#}$p=0.018, age group 2vs3 p=0.06) (**I**). One-way ANOVA and Student's *t*-test were applied and a *p* value <0.05 was considered statistically significant.

By stratifying the whole sample size according to gender, the β1 subunit showed a linear decrease with age in the liver of male donors (p=0.008) (**fig. 7B**), but not in female subjects (p=0.07) (**fig. 7A**).

We also calculated the ratio between each catalytic subunit and α4 subunit, obtaining an index of the proportion of constitutive and inducible subunits on total proteasomes (Rodriguez et al., 2010). Remarkably, an increase in β5i/α4 ratio in the whole sample size (p=0.0027) (**fig**. **7C**) and in biopsies of female (p=0.019) (**fig. 7D**) and male subjects (p=0.039) (**fig. 7E**) was observed. Regarding PA28αβ proteasomes regulators we confirmed that PA28α subunit decreased with age when biopsies from male (p=0.048) (**fig. 7G**), but not from female donors (**fig. 7F**), were considered.





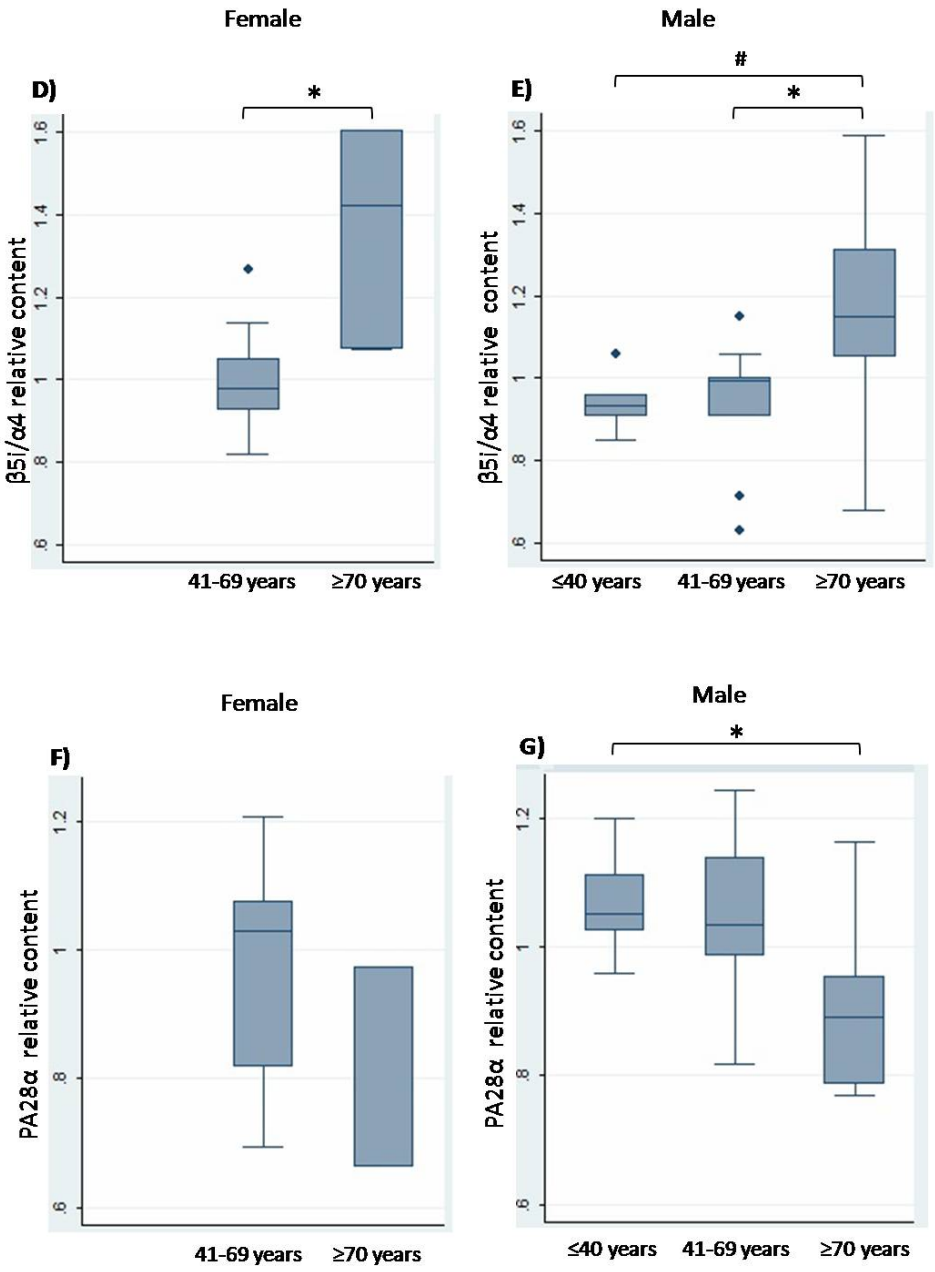


Figure 7. Relative content of proteasomes subunits in human liver of female and male subjects. Densitometric analysis of the expression levels of proteasomes subunits in crude protein extracts from human liver biopsies of females (F) and males (M) belonging to young (≤40 years, F n=0, M n=7), middle age (41-69 years, F n=16, M n=10), and elderly (≥70 years, F n=3, M n=6) subjects. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. An age-related decrease of β1 subunit was observed considering samples from male donors (one-way ANOVA $^{\bullet}$p=0.008; age group 1vs2 *p=0.027, age group 1vs3 $^{\#}$p=0.0021, age group 2vs3 p=0.24) (**B**) but not in those from females (Student's *t*-test p=0.07) (**A**). β5i/α4 ratio increased during aging in the whole cohort (Kruskal-Wallis test p=0.0027; Mann-Whitney *U* test age group 1vs2 p=0.26, age group 1v3 $^{\#}$p=0.045, age group 2vs3 *p=0.025) (**C**) and in both male (Kruskal-Wallis test p=0.039; Mann-Whitney *U* test age group 1vs2 p=0.38, age group 1v3 $^{\#}$p=0.013, age group 2vs3 *p=0.0017) (**E**) and female donors (Student's *t*-test *p=0.019) (**D**). A decreased PA28α content was observed in male donors subset (one way ANOVA

p=0.048; age group 1vs2 p=0.69, age group 1vs3 *p=0.030, age group 2vs3 p=0.05) (**G**) but not in female donors (Student's *t*-test p=0.32) (**F**). One-way ANOVA, Kruskal-Wallis, Mann-Whitney and Student's *t*-test were applied.

4.3 PROTEASOME SUBCELLULAR LOCALIZATION

We also performed **immunohistochemical analysis** to assess which cell types undergo the above mentioned age-related modifications and we extended our previous observations on subcellular distribution of proteasomes in human liver. As previously described (Vasuri et al., 2010; Gohlke et al., 2013), β1 and PA28α subunits were variably expressed both at nuclear and cytoplasmic level, in all cell types, including biliocytes; while β1i and β5i showed a cytoplasmic positivity in all cell types, in particular in lymphocytes and Kupffer cells. In the current work we focused on hepatocytes, which represent the main cell population of the liver. A strong (2+) nuclear positivity for β5i was recorded in 8 (15%) cases and for β1i just in 1 (2%) case, whereas a mild-to-moderate cytoplasmic positivity for both the subunits was observed in all cases. A strong nuclear immune-reactivity for β1 was seen in 47 (87%) cases, while cytoplasm was positive (2+) to β1 in 26 (48%) cases. The same distribution was observed in biopsies from female and male donors (data not shown). Notably, no changes in expression level and localization of β1, β1i, β5i subunits were observed in human hepatocytes during aging (**fig. 8a-f**). As far as it concerns PA28αβ proteasomes regulator, the PA28α subunit showed a nuclear and cytoplasmic positivity (2+) in 38 (70%) and 23 (43%) cases respectively (**table 9**). Remarkably, this positivity significantly decreased with aging (C=-0.343, p=0.011) (**fig. 8g, h**), confirming at least the loss of PA28α subunit recorded in crude protein extracts from male donors.

Immunohistochemical analysis		
Compartment positivity	cytoplasm	nucleus
β1	(2+) 48%	(2+) 87%
β1i	(+) 99%	(2+) 2%
β5i	(+) 99%	(2+) 15%
PA28α	(2+) 43%	(2+) 70%

Table 9. **Immunohistochemical analysis of proteasomes in human hepatocytes of liver biopsies (n=54)**. In Table are shown proteasomes subunits representative of proteasomes subcellular distribution at cytoplsmic and nuclear level. The positivity to each subunit is reported as strong (2+) or mild-to-moderate (+) and also the percentage frequency in all liver biopsies analyzed is shown.

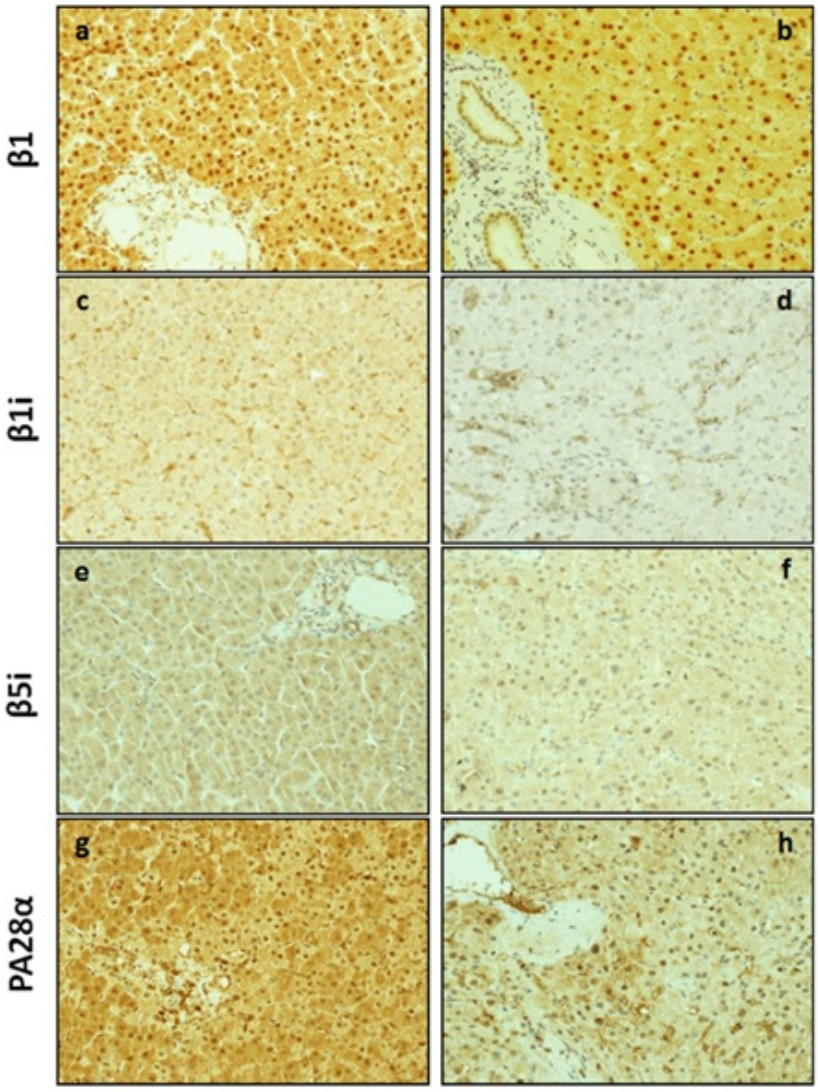


Figure 8. Immunohistochemical analysis of proteasomes distribution showed an age-related decrease of PA28α subunit in the citosol of hepatocytes. A young (on the left) and an old (on the right) liver donors are represented. β1 subunit (**a, b**) showed the same strong nuclear and cytoplasmic positivity, while β1i (**c, d**) and β5i (**e, f**) were always negative or weakly expressed in the nucleus. Note the decreased cytoplasmic positivity of PA28α in older livers (**h**), compared to younger (**g**) (Spearman test $^{\bullet}p=0.011$). 20x magnification.

4.4 PROTEASOME FUNCTIONALITY

4.4.1 OXIDIZED PROTEIN CONTENT

An increased level of **oxidized proteins** has been described in the liver of aged mice and it was directly correlated to an impairment in proteasomes activities (Breusing et al., 2009). We investigated whether these age-related modifications occur also in human liver. We measured the content of oxidized proteins in biotic samples from donors with different age by oxyblot detection kit (**fig. 9**).

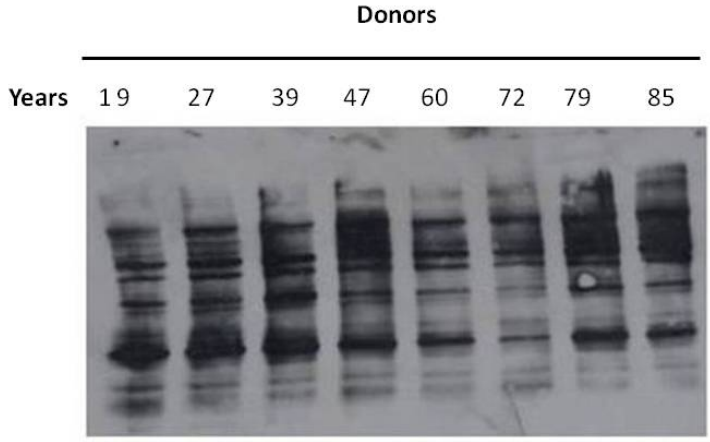


Figure 9. **Analysis of oxidized proteins by oxyblot detection in crude protein extracts from human liver biopsies of different age.** Equal amounts of crude protein extracts were analyzed by immunoblotting for oxidized proteins content. The picture shows a representative blot from liver samples with the characteristic smear of modified proteins. Protein isoloading was assessed by Coomassie gel staining (data not shown).

The densitometric quantification of the smears of oxyblots showed that no changes were observed (p=0.70) (**fig. 10**).

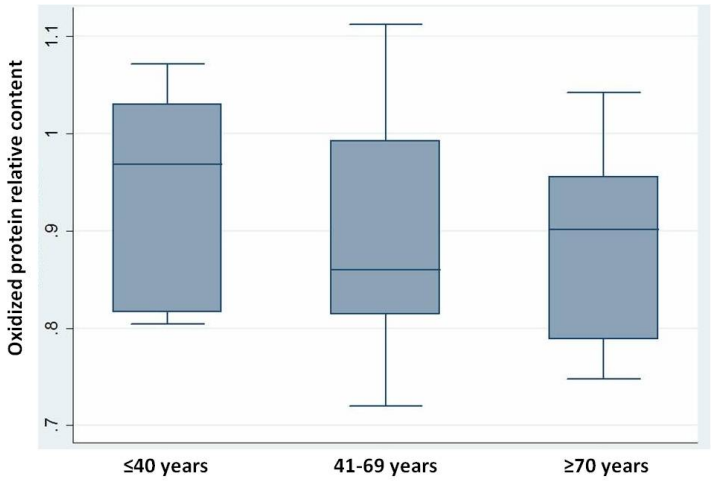


Figure 10. Relative content of oxidized proteins in human liver of young (≤40 years), middle age (41-69 years) and elderly (≥70 years) subjects. Western blotting analysis of oxidized proteins was performed on crude protein extracts from different age human livers (young n=7; middle age n=26; old n=9). Densitometric measure of the bands corresponding to oxidized proteins was considered and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference among age groups was analyzed using one-way ANOVA test and a *p* value<0.05 was considered statistically significant. The relative content of oxidized proteins in human liver was preserved during aging (p=0.70).

4.4.2 POLY-UBIQUITIN CONJUGATED PROTEIN CONTENT

We also analyzed the amount of **poly-ubiquitin conjugated proteins**, which represent another indirect index of proteasomes functionality (**fig. 11**), and even in this case no statistically significant differences among age groups emerged (p=0.19) (**fig. 12**).

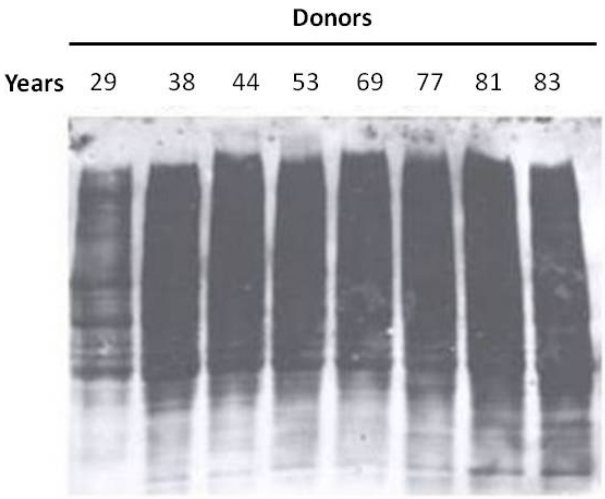


Figure 11. Content of mono- and poli-ubiquitin conjugated proteins in crude protein extracts of human liver from donors of different age. Representative immunobloting of liver crude protein extracts by using an antibody specific for K^{48}-, K^{63}- and K^{29}- linked conjugates, that recognizes mono- and poly-ubiquitin conjugated proteins, but not free ubiquitin molecules. A characteristic smear of modified proteins was observed and quantified. Protein isoloading was assessed by Coomassie gel staining (data not shown).

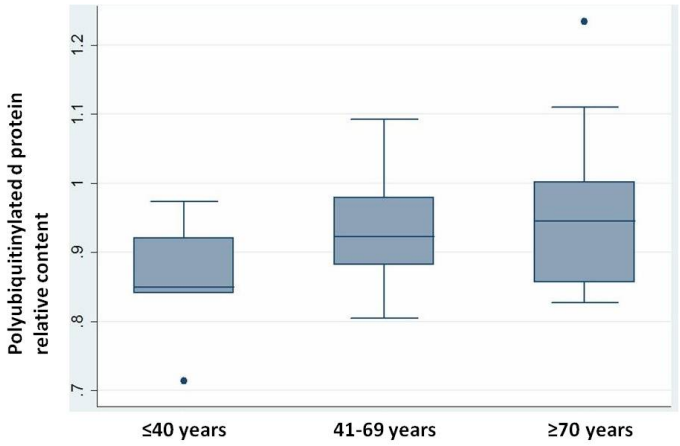


Figure 12. Relative content of poly-ubiquitinylated proteins in human liver of young (≤40 years), middle age (41-69 years) and elderly (≥70 years) subjects. Western blotting analysis of poly-ubiquitin conjugates was performed on crude protein extracts from different age human livers (young n=7; middle age n=26; old n=9). Densitometric measure of the bands corresponding to poly-ubiquitinylated proteins was considered and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference among age groups was analyzed using one-way ANOVA test and a *p* value<0.05 was considered statistically significant. The relative content of poly-ubiquitin conjugated proteins was preserved during aging (p=0.19).

In our study, gender does not seem to affect the levels of oxidized and poly-ubiquitin conjugated proteins (data not shown).

4.4.3 PROTEASOME ACTIVITY

The analysis of **proteasomes activities**, measured by short fluorogenic peptides assay and reported as specific activity for total protein (Rodriguez et al. 2010), showed that the CT-like (p=0.64) (**fig. 13A**) and the C-like (p=0.40) (**fig. 13C**) specific activities were preserved during aging, while a trend to increase was observed for the T-like specific activity (p=0.07) (**fig. 13B**). However, normalizing the three proteolytic activities for α4 subunit, a non-replaceable component of the catalytic core and thus representative of total proteasomes content, any difference emerged (**fig. 13D-F**).

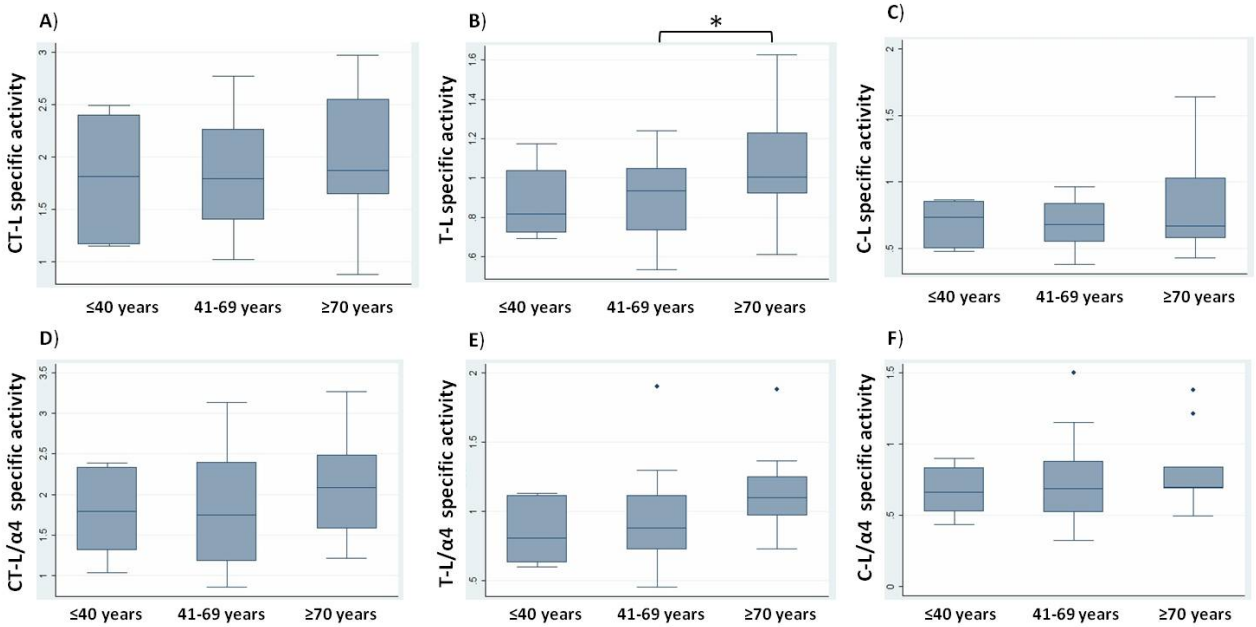


Figure 13. Proteasome functionality in human livers of young (≤40 years), middle age (41-69 years) and elderly (≥70 years) subjects. Proteasomes specific activities were measured in crude protein extracts from human liver biopsies (young n=7; middle age n=26; old n=9). Hydrolysis of the fluorogenic peptides LLVY-AMC (**A**), VGR-AMC (**B**) and LLE-AMC (**C**) measured the chymotripsin-like (CT-L), the trypsin-like (T-L) and the caspase-like (C-L) activities, respectively. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference among age groups was analyzed by using one-way ANOVA and Student's *t*-test and a *p* value<0.05 was considered statistically significant. None of the three proteasome activities (expressed as nmol/mg*min) was affected by aging, even if a trend to increase was observed for T-L activity (one-way ANOVA p=0.07; Student's *t*-test age group 1vs2 p=0.91, age group 1vs3 p= 0.16, age group 2vs3 *p=0.04) (**B**). The same results emerged dividing specific activities by α4 relative content: CT-L/ α4 p= 0.49 (**D**), T-L/ α4 p= 0.11 (**E**), C-L/ α4 p=0.40 (**F**).

Of note, by stratifying our data according to gender we observed that proteasomes activities were differently affected by aging, in the liver of men and women.

In fact, only in liver from male donors, the T-like activity/α4 relative content was increased in the old group compared to the middle-age group (p=0.014), with no statistically significant differences between young and old age groups (p=0.46) (**fig. 14B**).

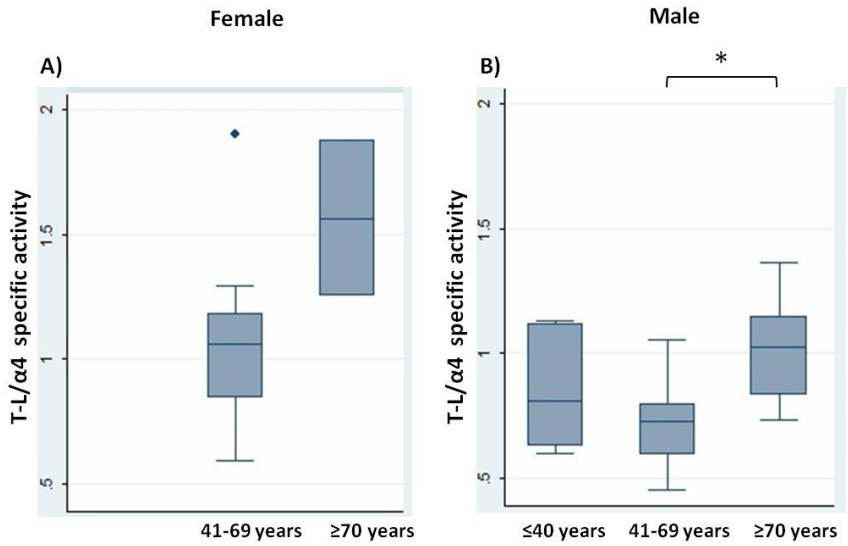


Figure 14. Proteasome trypsin-like/α4 specific activity in human livers of female and male subjects. Proteasome trypsin-like specific activity was measured by using VGR-AMC substrate in crude protein extracts from biopsies of females (F) and males (M) belonging to young (≤40 years, F n=0, M n=7), middle age (41-69 years, F n=16, M n=10), and elderly (≥70 years, F n=3, M n=6) subjects and normalized for α4 relative content, measured by western blotting. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference among age groups was analyzed using one-way ANOVA test and Student's *t*-test and a *p* value<0.05 was considered statistically significant. Only in males (**B**), but not in females (**A**) a non-monotonic age-related modification of the trypsin-like activity per amount of α4 relative content was observed. In particular in male group the comparison between the middle-age and the elderly subjects (*p=0.014) was statistically significant.

Similarly, a decline in the C-like activity/α4 relative content in the male middle-age group was recorded, along with no differences between the younger and the older subjects (p=0.026) (**fig. 15**).

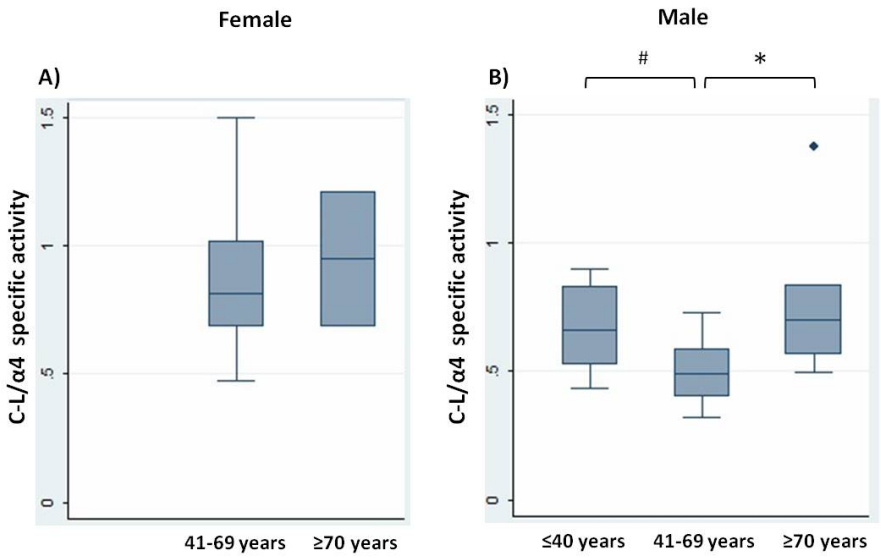


Figure 15. Proteasome caspasic-like/α4 specific activity in human livers of female and male subjects. Proteasome caspasic-like specific activity/α4 relative content was measured by using LLE-AMC substrate in crude protein extracts from biopsies of females (F) and males (M) belonging to young (≤40 years, F n=0, M=7), middle age (41-69 years, F n=16, M n=10), and elderly (≥70 years, F n=3, M n=6) subjects and normalized for α4 relative content, measured by western blotting. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference among age groups was analyzed by using one-way ANOVA test and Student's *t*-test and a *p* value<0.05 was considered statistically significant. Only in males (**B**), but not in females (**A**) a non-monotonic age-related modification of the caspasic-like activity per amount of α4 relative content was observed (one-way ANOVA •p=0.026; Student's *t*-test age group 1vs2 $^{\#}$p=0.034, age group 1vs3 p=0.38, age group 2vs3 *p=0.014).

4.A/B RESULTS ON HUMAN SKELETAL MUSCLE

4.A ANALYSES ON BIOPSIES OF VASTUS LATERALIS SKELETAL MUSCLE FROM HEALTHY SUBJECTS AND PATIENTS WITH LOWER LIMB MOBILITY IMPAIRMENT (LLMI)

4.A1 EXPRESSION LEVELS OF PROTEASOME SUBUNITS

Many efforts have been done to understand the contribution of the UPS, one of the major pathways for protein degradation, to the loss of muscle mass that occurs during aging in both human and rodents. To date, most of the studies were performed in animal models and were mainly focused on ubiquitin pathway rather than proteasomes. Indeed, few data are available on the contribution and involvement of proteasomes, and in particular of i-proteasome, in the aging of human skeletal muscle.

In the framework of the European project MYOAGE ''Understanding and Combating Age-related Muscle weakness", we collected biopsies of *v. lateralis* skeletal muscle from hospitalized patients (Rizzoli Hospital, Bologna) with limited lower limb mobility impairment (LLMI patients) undergoing surgery for hip replacement, and healthy physically and cognitively active subjects.

We firstly analyzed proteasomes composition and content, **at mRNA level**, in *v. lateralis* muscle biopsies obtained from female healthy subjects and LLMI patients, both belonging to different age. In particular we focused our attention on α6 subunit, which is a not-replaceable component of the catalytic core and thus representative of total proteasomes content, on β1 catalytic subunit of constitutive proteasomes, β1i and β5i catalytic inducible subunits of i-proteasomes as well as the inducible subunit PA28β, representative of PA28αβ proteasomes regulator.

Quantitative PCR (qPCR) analysis showed that all the constitutive and inducible components of proteasomes were present at transcriptional level in *v. lateralis* muscle of healthy subjects and LLMI patients.

Additionally, in **healthy subjects** we found a statistically significant age-related increase in the expression level of the inducible subunits β1i (p=0.009) (**fig. 16D**) and β5i (p=0.009) (**fig. 16E**), as well as PA28β (p=0.036) (**fig. 16C**), while the constitutive subunits α6 and β1 were not affected by aging (p=0.173 and p=0.072 respectively) (**fig. 16A-B**).

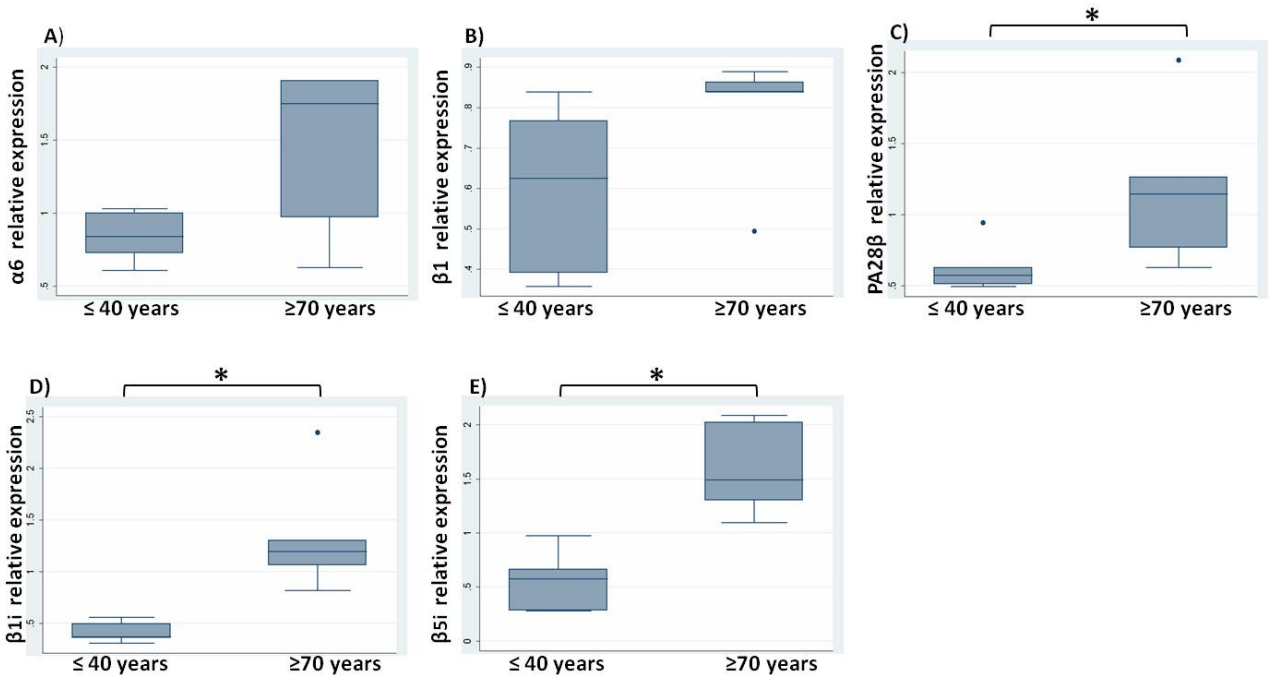


Figure 16. Quantitative PCR analysis of mRNAs encoding proteasomes subunits in human *v. lateralis* muscle of young (≤40 years, n=5) and elderly (≥70 years, n=5) female healthy subjects. Relative expression of mRNA levels was measured by using specific primers for α6 and β1 constitutive subunits (**A**, **B**), PA28β inducible subunit (**C**), β1i and β5i i-proteasome subunits (**D**, **E**). We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference between the two age groups was analyzed using Mann-Whitney test and a *p* value<0.05 was considered statistically significant. An age-related increase in the expression levels of inducible subunits PA28β (**C**, *p=0.036), β1i (**D**, *p=0.009) and β5i (**E**, *p=0.009) was observed, while the α6 and β1 constitutive subunits were not affected by aging (**A**, p=0.173 and **B** p=0.072 respectively).

On the contrary, when we analyzed the expression levels of these proteasomes subunits in **LLMI patients**, no statistically significant differences were observed between age groups, even if a general trend to increase was present (**fig. 17A-E**).

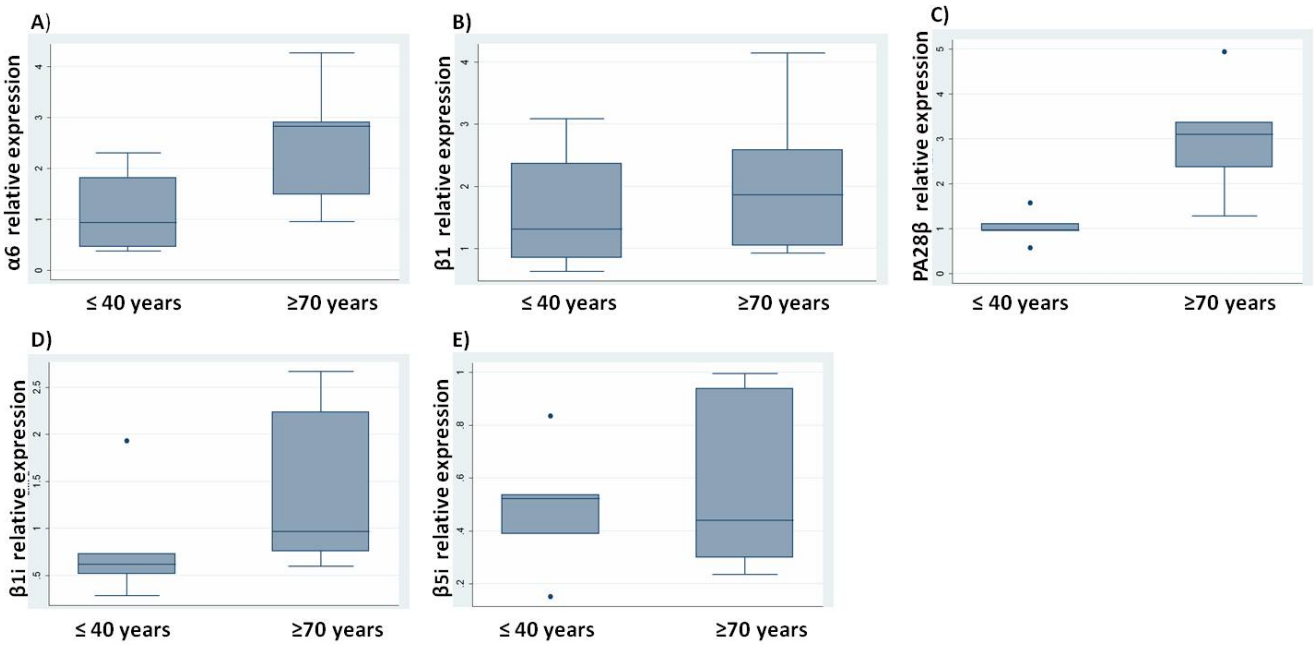


Figure 17. Quantitative PCR analysis of mRNAs encoding proteasomes subunits in human *v. lateralis* muscle of young (≤40 years, n=6) and elderly (≥70 years, n=6) female LLMI patients. Relative expression of mRNA levels was measured by using specific primers for α6 and β1 constitutive subunits (**A**, **B**), inducible subunit PA28β (**C**), as well as β1i and β5i i-proteasome subunits (**D**, **E**). We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Difference between the two age groups was analyzed by using Student's *t* (for β1, α6, PA28β, β5i) and Mann-Whitney (for β1i) tests and a *p* value<0.05 was considered statistically significant. No age-related modification in the expression levels of these proteasomes subunits in LLMI patients was observed.

4.A2 PROTEASOME COMPOSITION AND CONTENT

We analyzed by western blotting proteasomes composition and content **at the protein level**. Experiments performed on crude protein extracts, from **healthy female subjects**, showed that the constitutive proteasome represents the major population in human *v. lateralis* skeletal muscle, while the inducible components of i-proteasome, β1i and β5i, as well as PA28β subunit, were undetectable or absents (**fig. 18a, b**).

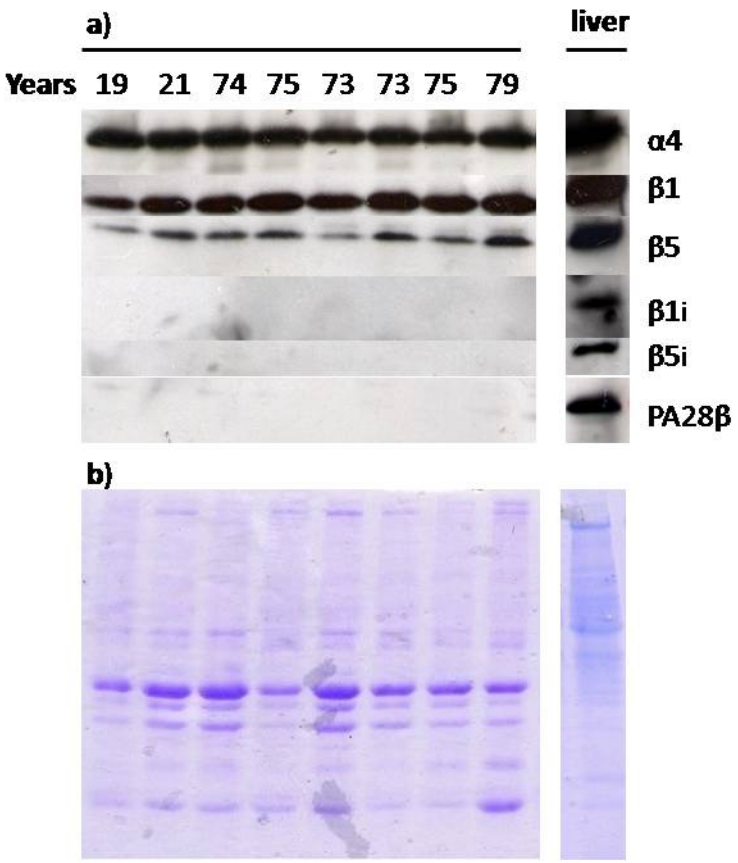


Figure 18. Western blotting analysis of crude protein extracts from human *v. lateralis* muscle of female healthy subjects. α4, β1 and β5 constitutive subunits are expressed in both young and elderly healthy subjects, while β1i, β5i and PA28β inducible subunits are almost undetectable compared to a liver sample, used as internal positive control for proteasomes subunits (**a**). Protein isoloading was assessed by Coomassie gel staining (**b**).

This suggests that post-transcriptional mechanisms might impinge upon protein synthesis, assembly and/or maturation of inducible proteasomes components in healthy subjects.

We next performed a densitometric analysis of the bands corresponding to proteasomes subunits obtained by western blotting, in order to assess possible age-related modification in human *v. lateralis* muscle of healthy subjects. Thus, taking into account that the signal of the bands corresponding to inducible components of proteasomes was weak for a reliable quantitative estimation, we performed a densitometric analysis only on constitutive components of proteasomes, in particular on α4, β1 and β5. Additionally, since some free α subunits might be found in cells, in particular during senescence (Chondrogianni et al., 2005) we also have calculated the ratio between each catalytic subunit and α4 (β1/α4 and β5/α4 ratio), obtaining an index of assembled proteasomes (Rodriguez et al., 2010).

In **healthy female subjects** we found that the content of α4 and β1 subunits and of the ratio β1/α4 were unchanged during aging (p=0.81, p=0.82 and p=0.78 respectively) (**fig. 19A, B, D**), while the level of β5 catalytic subunit and the ratio β5/α4 were increased (p=0.037 and p=0.047, respectively) (**fig. 19C, E**).

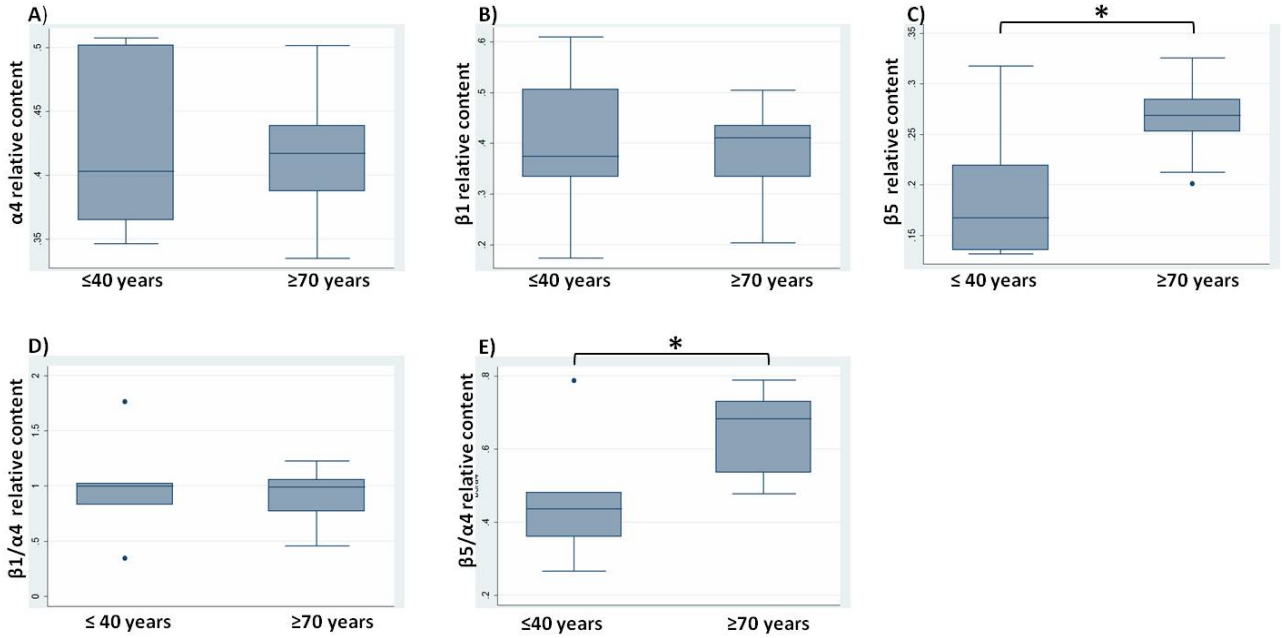


Figure 19. Relative content of proteasomes constitutive subunits in crude protein extracts from human *v. lateralis* muscle of young (≤40 years, n=5) and elderly (≥70 years, n=9) healthy subjects. Densitometric analysis of the bands corresponding to α4 (**A**), β1 (**B**) and β5 (**C**) constitutive subunits was performed and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. The ratio between each catalytic subunit and α4 (β1/α4, β5/α4), was also calculated (**D**, **E**). Difference between the two age groups was analyzed using Student's *t*-test and a *p* value<0.05 was considered statistically significant. An age-related increase of β5 relative content (**C**, *p=0.037) and of β5/α4 ratio (**E**, *p=0.047) was observed.

Of note, these findings also emerged when we investigated proteasomes protein composition of human *v. lateralis* muscle of female and male **LLMI patients**. Indeed inducible components β1i and PA28β were barely detectable, while β5i was undetectable or absent (**fig 20a, b**).

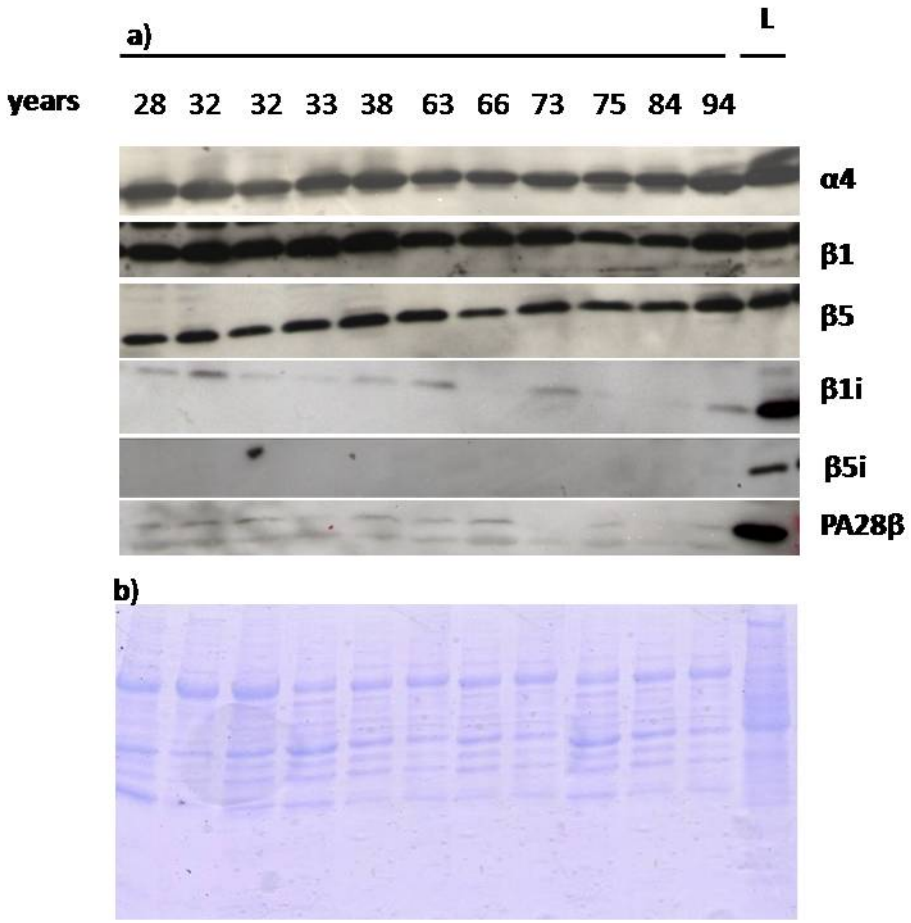


Figure 20. **Western blotting analysis of crude protein extracts from human *v. lateralis* muscle of LLMI patients**. α4, β1 and β5 constitutive subunits are expressed in LLMI patients of different age, while β1i, β5i and PA28β inducible subunits are almost undetectable compared to a liver sample (L), used as internal positive control for proteasomes subunits (**a**). Protein isoloading was assessed by Coomassie gel staining (**b**).

In **LLMI patients**, the same analysis showed that proteasomes content was largely preserved during aging (**fig. 21A-D**), indeed only a borderline non-monotonic variation in the β5/α4 ratio (p=0.06) (**fig. 21E**) was observed. In particular, an age-related decrease in elderly (≥70 years) respect to middle-age (41-69 years) patients was found (p=0.039), while the young group and the old one did not differ.

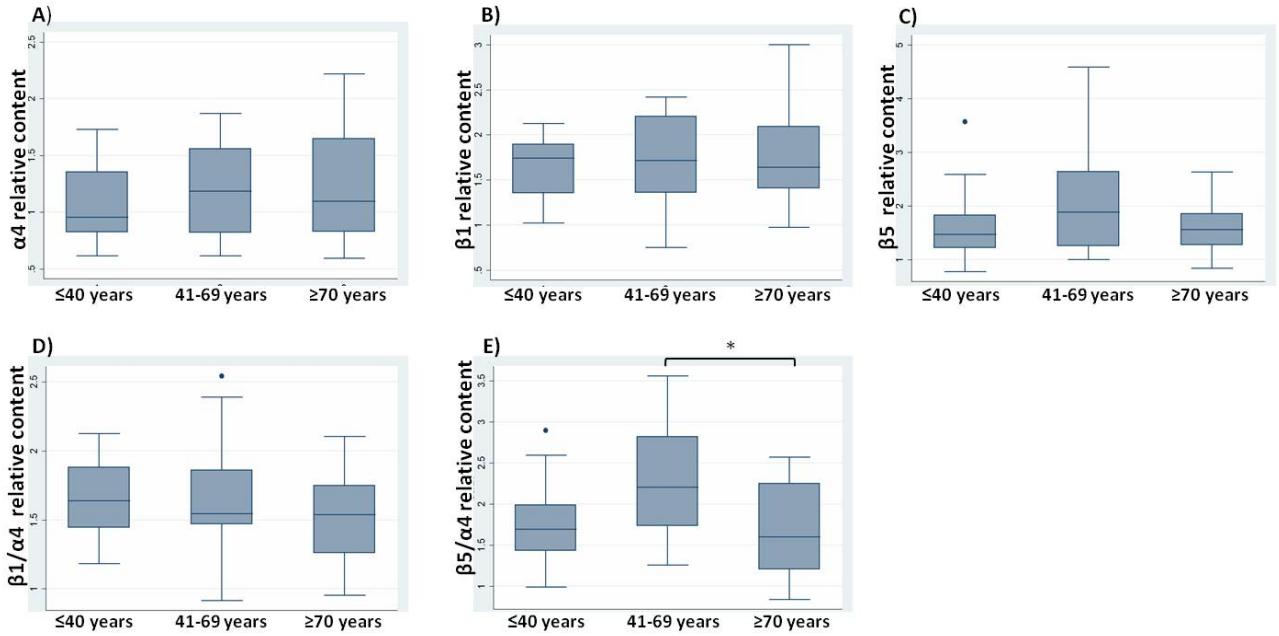


Figure 21. Relative content of proteasomes constitutive subunits in crude protein extracts from human *v. lateralis* muscle of young (≤40 years, n=14), middle age (41-69 years, n=18) and elderly (≥70 years, n=17) LLMI patients. Densitometric analysis of the bands corresponding to α4 (**A**), β1 (**B**) and β5 (**C**) constitutive subunits was performed and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. The ratio between each catalytic subunit and α4 (β1/α4, β5/α4) was also calculated (**D**, **E**). Differences among the three age groups were analyzed by using Kruskal-Wallis test and a *p* value<0.05 was considered statistically significant. Only an age-related decrease of β5/α4 relative content was observed, comparing the group of middle-age to the elderly subjects (**E**, *p=0.039).

This latter result also emerged when muscle samples from female **LLMI patients** were considered (**fig 22A, B**); in particular a decrease of β5/α4 ratio was observed in the old group compared to the middle-age patients (p=0.022) (**fig. 22A**). Remarkably, no age-related modification of proteasomes content was observed in male LLMI patients.

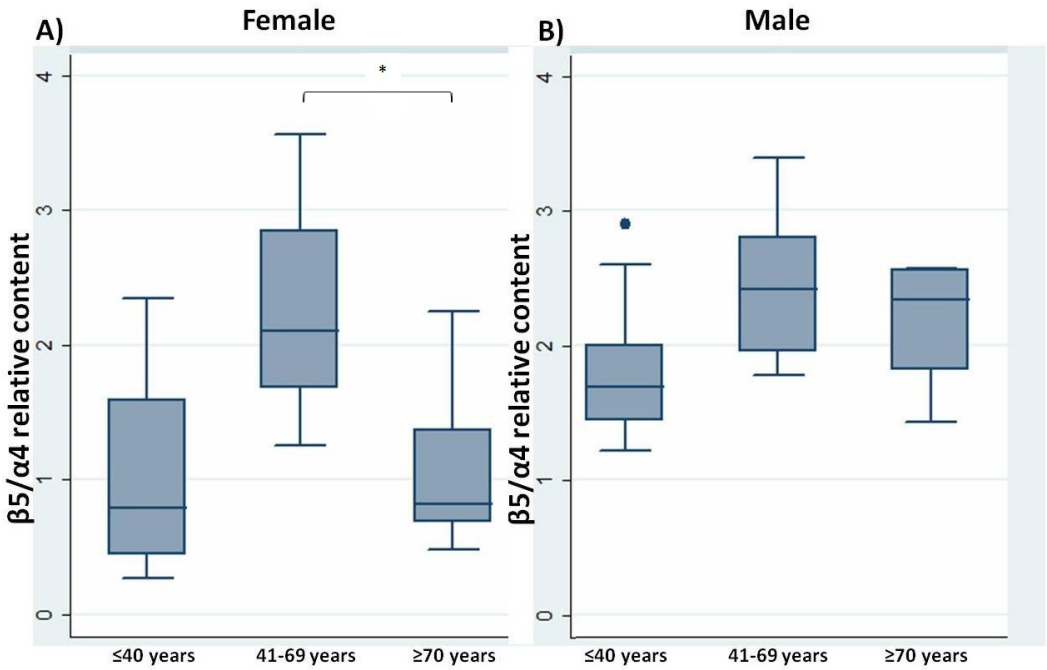


Figure 22. Relative content of β5/α4 ratio in crude protein extracts from human *v. lateralis* muscle of young (≤40 years), middle age (41-69 years) and elderly (≥70 years) female and male LLMI patients. Densitometric analysis of β5/α4 relative content in crude protein extracts from human *v. lateralis* biopsies of females (F) and males (M) belonging to young (≤40 years, F n=5, M n=9), middle age (41-69 years, F n=12, M n=6), and elderly (≥70 years, F n=11, M n=6) LLMI patients. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Differences among the three age groups were analyzed by using Kruskal-Wallis test and a *p* value<0.05 was considered statistically significant. The comparison between the middle-age subjects and the elderly in female patients was statistically significant (**A**, *p=0.022), while no age-related modification was observed in male patients (**B**).

Finally, we investigated whether muscle inactivity affected proteasomes content in human skeletal muscle, by comparing healthy subjects and LLMI patients. This analysis was possible only for female subjects because muscle biopsies from healthy male donors were not available (**fig. 23**).

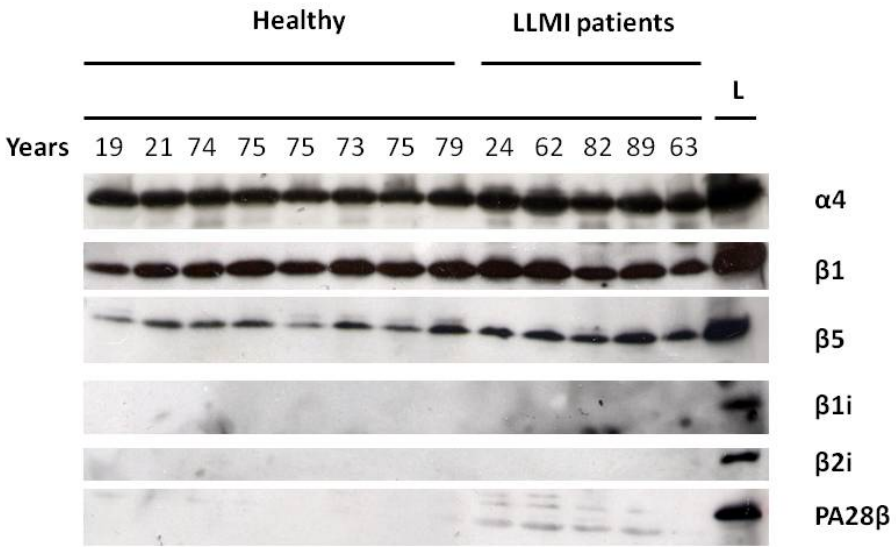


Figure 23. Western blotting analysis of crude protein extracts from human *v. lateralis* muscle of female healthy subjects and LLMI patients. No difference in proteasomes composition was observed between healthy subjects and LLMI patients. Indeed, the constitutive subunits α4, β1 and β5 were expressed in both young and elderly healthy subjects and LLMI patients, while the inducible subunits β1i, β5i and PA28β were almost undetectable, compared to a liver sample (L), used as internal positive control for proteasomes subunits. Protein isoloading was assessed by Coomassie gel staining (data not shown).

Remarkably, semi-quantitative densitometric analyses showed that independently of the age of subjects, all the proteasomes subunits considered were present at significant higher levels in LLMI patients that in healthy subjects (α4 p=0.0002; β1 p=0.0001; β5 p=0.0001; β1/α4 p=0.0073; β5/α4 p=0.0001) (**fig. 24A-E**).

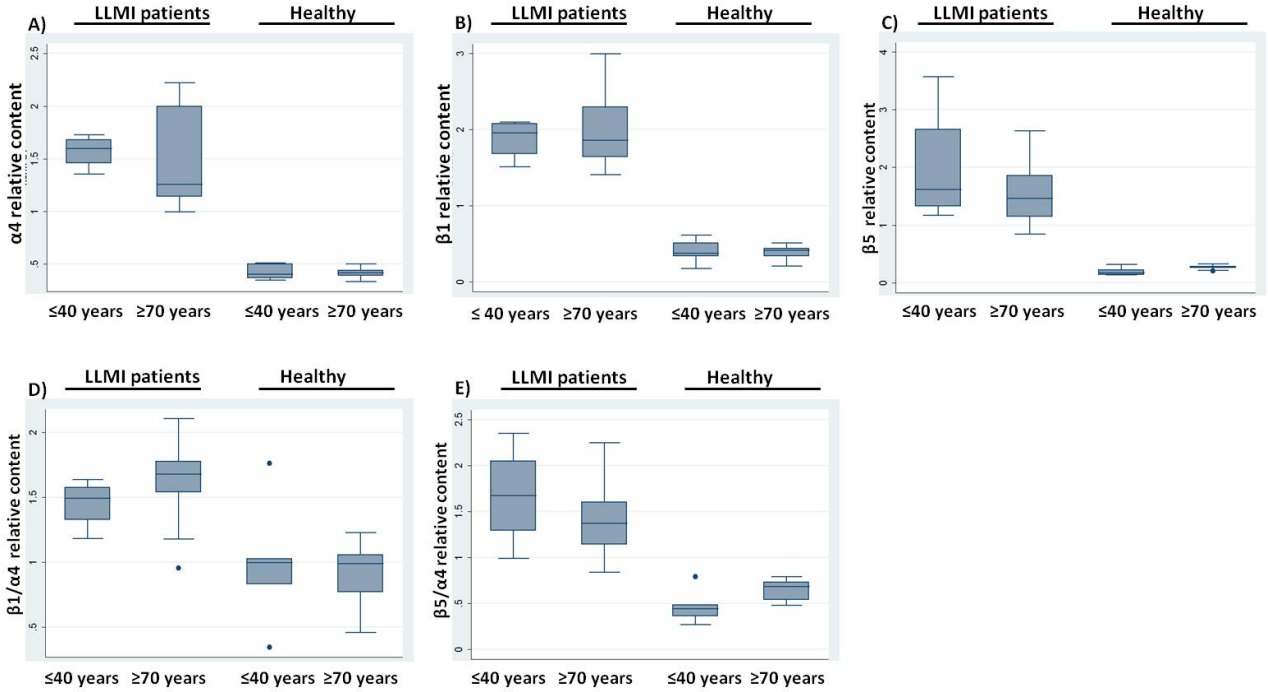


Figure 24. Comparison of the relative content of proteasomes constitutive subunits in human *v. lateralis* muscle of young (≤40 years) and elderly (≥70 years) healthy donors and LLMI patients. Densitometric analysis of the bands corresponding to the constitutive subunits α4 (**A**), β1 (**B**) and β5 (**C**) was performed in crude protein extracts from human *v. lateralis* muscle biopsies of young (≤40 years, healthy n=5, LLMI n=14) and elderly (≥70 years, healthy n=14, LLMI n=17) subjects. Data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. The ratio between each catalytic subunit and α4 (β1/α4, β5/α4) was also calculated (**D**, **E**). Differences between groups were analyzed by using Kruskal-Wallis test and a *p* value<0.05 was considered statistically significant. Independently from age, all the proteasomes subunits considered were present at significant higher levels in LLMI patients than in healthy subjects (**A**, α4 $^{\bullet}$p=0.0002; **B**, β1 $^{\bullet}$p=0.0001; **C**, β5 $^{\bullet}$p=0.0001; **D**, β1/α4 $^{\bullet}$p=0.0073; **E**, β5/α4 $^{\bullet}$p=0.0001).

4.A3 PROTEASOME SUBCELLULAR LOCALIZATION

Considering that the results on proteasomes composition seem to be almost the same in healthy subjects and LLMI patients, we took advantage of the larger amount of biopsies available from LLMI patients to deeply examine the subcellular distribution of proteasomes. Different cells constitute the mature skeletal muscle, such as myofibers (multinucleated syncytia of rhabdomyocytes which derive from mononuclear precursor cells), blood vessel endothelial cells

(whose proportion is different in type I and type II fibers), satellite cells (the stem cells reservoir of mature skeletal muscle) and cells of immune system (**fig. 25A**). Immunohistochemical analyses were performed by using antibodies specific for the constitutive (β1) and inducible (β1i and β5i) catalytic subunits.

As far as the β1 constitutive subunit, it was present in all cell types as expected and focusing on rhabdomyocytes we observed that it was mainly localized at cytosolic level in correspondence of the Z disc and of the A band of sarcomere (**fig. 25B**).

On the contrary, the inducible subunits β5i (**fig. 25C**) and β1i (**fig. 25D**) gave a weak or absent immune-staining in rhabdomyocytes at cytosolic level, while nuclei were almost negative. As reported in literature the endothelial cells, the white blood cells within vessels, and the tissue lymphocytes were always positive for i-proteasome (**fig. 25E**).

No difference on proteasomes staining was observed between young and elderly LLMI patients.

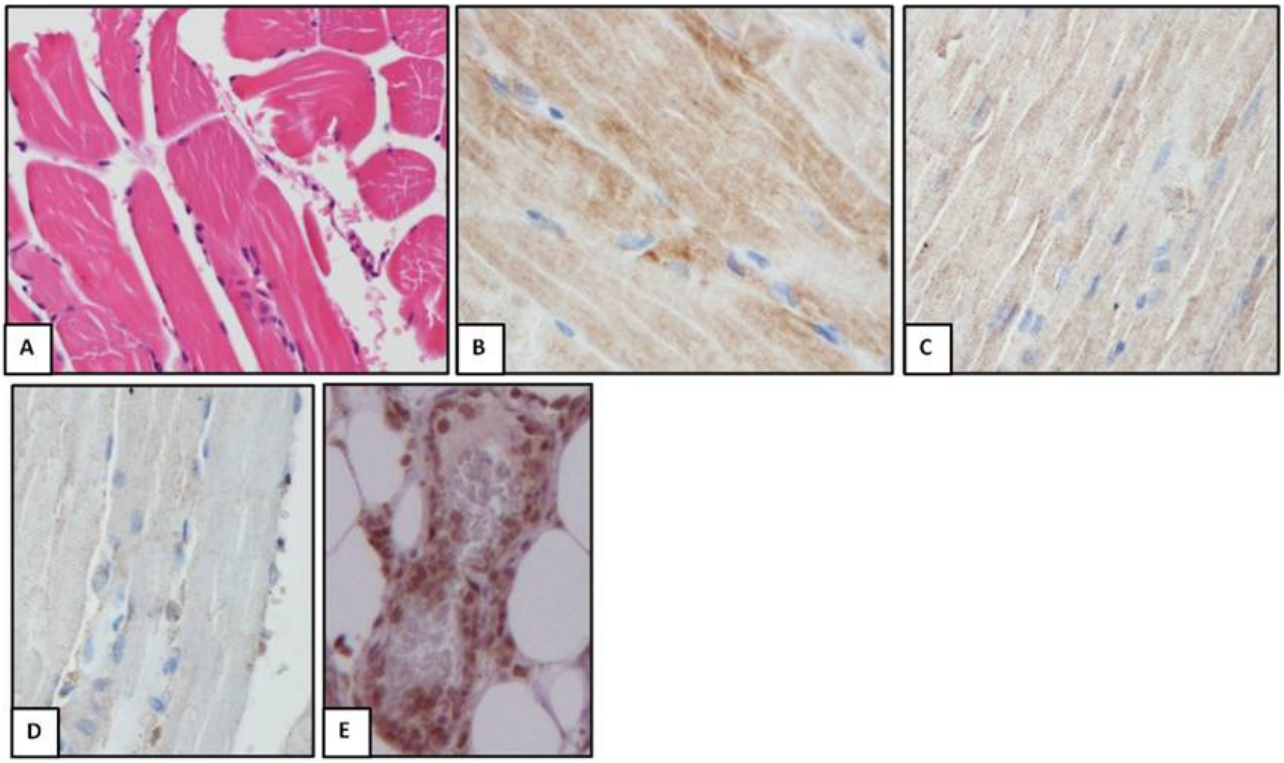


Figure 25. Proteasomes subcellular distribution in human *v. lateralis* muscle of LLMI patients. Immunohistochemical analyses on 3µm thick sections obtained from human *v. lateralis* muscle of LLMI patients of different age (young n=8 and old n=7 LLMI patients), were performed by using specific antibodies against the constitutive subunit β1 (**B**) and the inducible subunits β5i (**C**), β1i (**D**). Representative sections from one young LLMI patient (25 years) are shown. The β1 subunit showed strong positivity in the cytosol of rhabdomyocytes (**B**). On the contrary, the inducible subunits gave a weak (β5i, **C**) or absent (β1i, **D**) immune-staining in rhabdomyocytes at cytosolic level, while nuclei were almost negative. As expected the endothelial cells, the white blood cells within vessels, and the tissue lymphocytes were always positive for i-proteasome (**E**). Hematoxylin-eosin staining was also performed in order to highlight cellular compartment (**A**).

4.A4 PROTEASOME COMPOSITION AND CONTENT IN HUMAN MYOBLAST CELLS

Satellite cells are a population of mononuclear muscle stem cells that reside at the periphery of muscle fibers, between the basal lamina and the sarcolemma and play a pivotal role in muscle repair and regeneration. Actually, satellite cells are activated in response to different physiological and pathological stimuli to generate a committed population of myoblasts, that are capable of differentiate (Carosio et al., 2011).

Satellite cells were isolated from human *v. lateralis* muscle biopsies of young and elderly healthy subjects in the framework of the European project MYOAGE, and myoblast cell cultures were propagated at different oxygen tensions. We performed western blotting analysis on crude protein extracts, in order to assess whether proteasomes composition in muscle cell precursors is different from that observed *ex vivo* in mature and differentiated muscle cells. From this analysis we found that myoblasts expressed high level of i-proteasome subunits as well as PA28αβ regulator, concomitantly with constitutive proteasomes. Additionally, in our experimental conditions, despite variability among subjects, oxygen tension and age of the donor did not affect proteasomes composition and content (**fig. 26**).

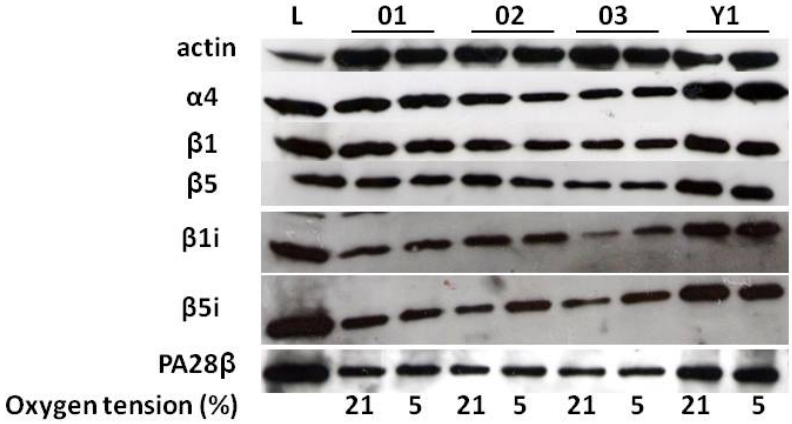


Figure 26. Representative immunoblotting of myoblast cell cultures isolated from human *v. lateralis* muscle of young and elderly healthy donors. Western blotting analysis was performed on crude protein extracts of myoblast cell cultures obtained from young (Y, n=3) and old (O, n=5) healthy subjects and propagated at different oxygen tensions (5% and 21%). Myoblasts expressed high level of the i-proteasome subunits β1i, β5i as well as PA28β of PA28αβ regulator, concomitantly with the constitutive subunits α4, β1 and β5. Oxygen tension and age of the donor did not affect proteasomes composition and content. A liver sample (L) was used as internal positive control for proteasomes subunits.

4.A5 PROTEASOME FUNCTIONALITY

Taking into account the subtle age-related modifications of proteasomes content observed in both healthy subjects and LLMI patients, we performed additional investigations to test whether aging impinged upon proteasomes functionality. Accordingly, we measured proteasomes

activity indirectly by the quantification of oxidized proteins and poly-ubiquitin conjugated content and directly by short fluorogenic peptides assay. All the analyses were performed in the LLMI patients of different age and gender, because no sufficient bioptic muscle samples from healthy donors were available.

4.A5.1 OXIDIZED PROTEIN CONTENT

As indirect marker of proteasomes functionality, we examined the content of oxidized proteins in human skeletal muscle of LLMI patients of different age by oxyblot detection kit (**fig. 27**).

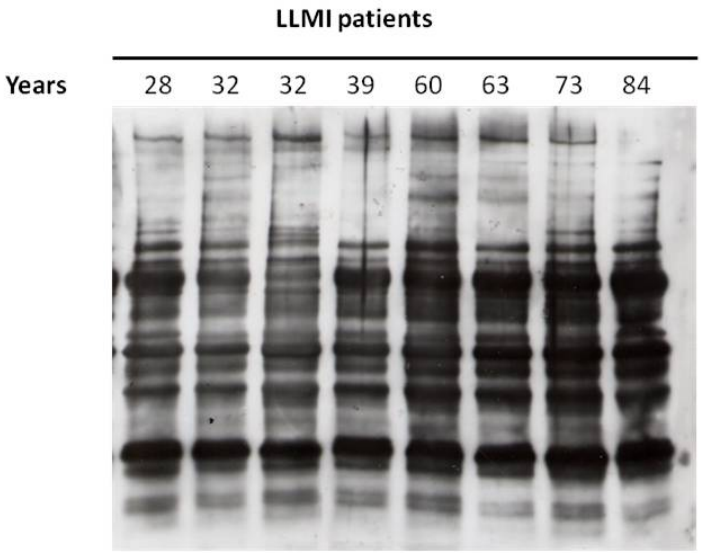


Figure 27. Analysis of oxidized proteins by oxyblot detection in crude protein extracts from human *v. lateralis* biopsies of LLMI patients of different age. Equal amounts of crude protein extracts were analyzed by immunoblotting for oxidized proteins content. The picture shows a representative blot from male muscle samples with the characteristic smear of modified proteins. Protein isoloading was assessed by Coomassie gel staining (data not shown).

The densitometric quantification of the smears of oxyblots showed that the levels of oxidized proteins were not modified during aging in whole samples size (p=0.11) (**fig. 28A**); on the contrary stratifying the results by gender, we found a slight age-related increase in oxidized proteins content in males (p=0.033) (**fig 28C**), but not in females (p=0.78) (**fig. 28B**).

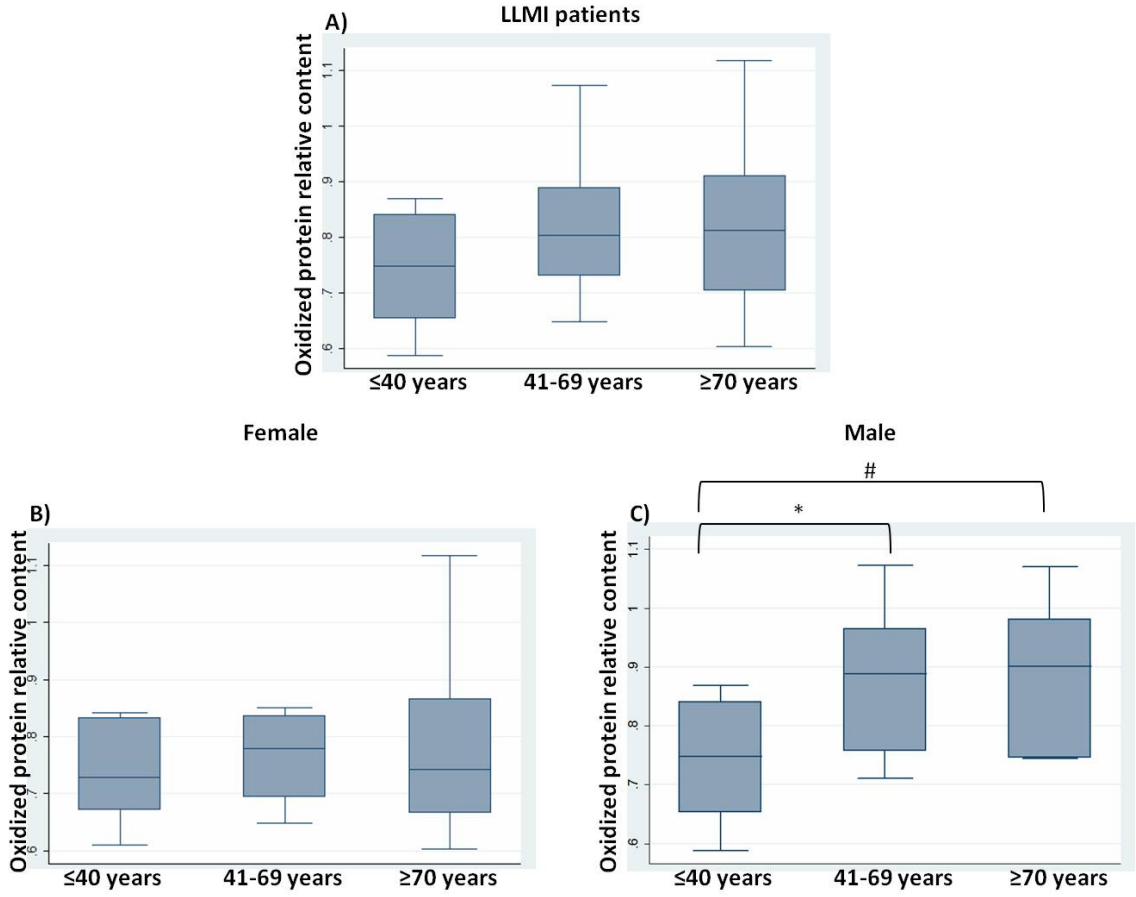


Figure 28. Relative content of oxidized proteins in human *v. lateralis* muscle of young (≤40 years), middle age (41-69 years), and elderly (≥70 years) LLMI patients. Densitometric analysis of the bands corresponding to oxidized proteins was performed and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Differences among age groups were analyzed by using ANOVA and Student's *t* tests and a *p* value<0.05 was considered statistically significant. The relative content of oxidized proteins in whole sample size (young n=15; middle-age n=25; old n=15) was preserved during aging (**A**, p=0.11). By stratifying data according to gender only in males (young n=9; middle-age n=12; old n=6) (**C**, p=0.033), but not in female subjects (young n=6; middle age n=13; old n=9) (**B**, p=0.78) an age-related increase of oxidized proteins content was observed. In particular, in males the comparisons between the young group and the middle-age one, and between young and elderly subjects were statically significant (**C**, *p=0.014, $^{\#}$p=0.035, respectively).

4.A5.2 POLY-UBIQUITIN CONJUGATED PROTEIN CONTENT

Proteins targeted to proteasome degradation are in general tagged by poly-ubiquitin chains that allow their recognition by 19S regulator of 26 proteasome. Thus, levels of poly-ubiquitin conjugated proteins may represent an indirect index of proteasomes functionality.

By using an antibody specific for K^{29}-, K^{48}- and K^{63}- linked conjugates (**fig. 29**), we found no age-related modification in the content of poly-ubiquitin conjugated proteins in the whole sample size (p=0.99) (**fig. 30A**), nor in females (p=0.33) (**fig. 30B**) nor in male patients (p=0.31) (**fig. 30C**).

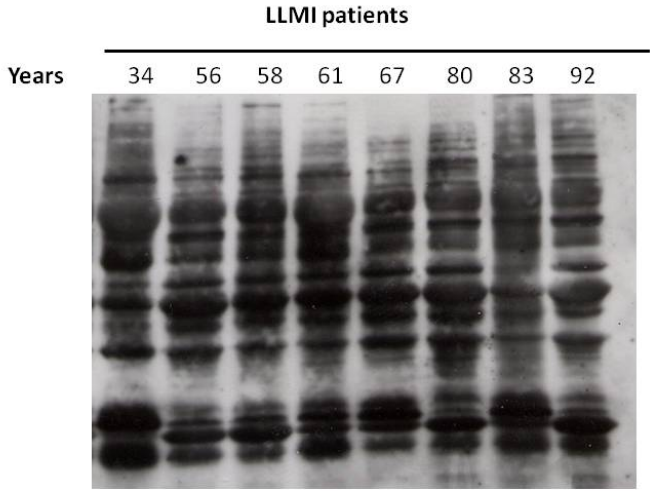


Figure 29. Content of mono- and poli-ubiquitin conjugated proteins in crude protein extracts of human *v. lateralis* muscle derived from LLMI patients of different age. Representative immunobloting of muscle crude protein extracts by using an antibody specific for K^{48}-, K^{63}- and K^{29}- linked conjugates, that recognizes mono- and poly-ubiquitin conjugated proteins, but not free ubiquitin molecules. A characteristic smear of modified proteins was observed and quantified. Protein isoloading was assessed by Coomassie gel staining (data not shown).

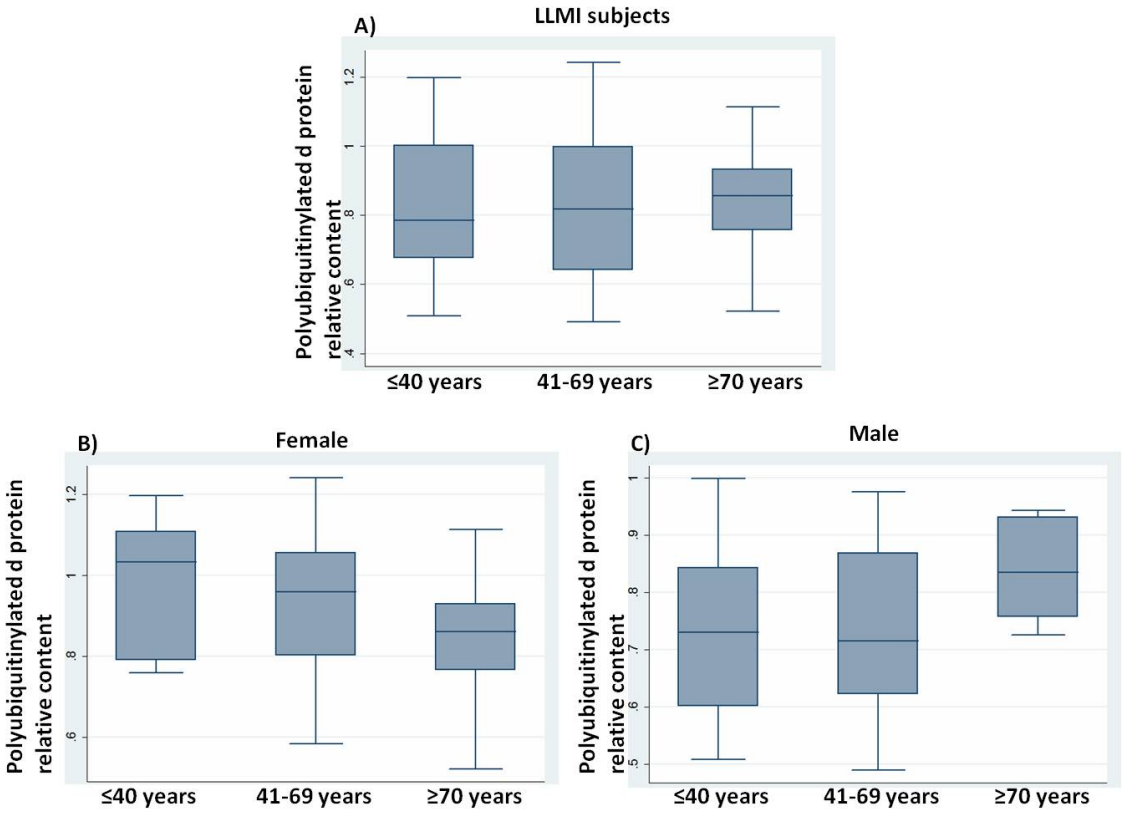


Figure 30. Relative content of poly-ubiquitin conjugated proteins in crude protein extracts of human *v. lateralis* muscle of young (≤40 years), middle age (41-69 years), and elderly (≥70 years) LLMI patients. Densitometric analysis of the bands corresponding to poly-ubiquitin conjugated proteins was performed and data were normalized for a sample used as internal control and loaded in every gel. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Differences among age groups were analyzed by using ANOVA test and a *p* value<0.05 was considered statistically significant. The relative content of poly-ubiquitin conjugated proteins in LLMI subjects (young n=16; middle-age n=22; old n=15) was not affected by aging (**A**, p=0.99). The same result emerged when females (young n=6; middle-age n=12; old n=9) (**B**, p=0.33) and male patients (young n=10; middle-age n=10; old n=6) (**C**, p=0.31) were analyzed separately.

4.A5.3 PROTEASOME ACTIVITY

Analyzing proteasomes functionality by short fluorogenic peptides assay in crude protein extracts of human skeletal muscle biopsies of LLMI patients, we found that the chymotrypsin-like (CT-L), trypsin- like (T-L) and caspase-like (C-L) specific activities were similar among the three age groups (p=0.85, p=0.37, p=0.78 respectively) (**fig. 31A-C**).

The same result emerged normalizing the three specific activities for α4 subunit relative content (CT-L/α4 p=0.63, T-L/α4 p=0.51, C-L/α4 p=0.78) (**fig. 31D-F**).

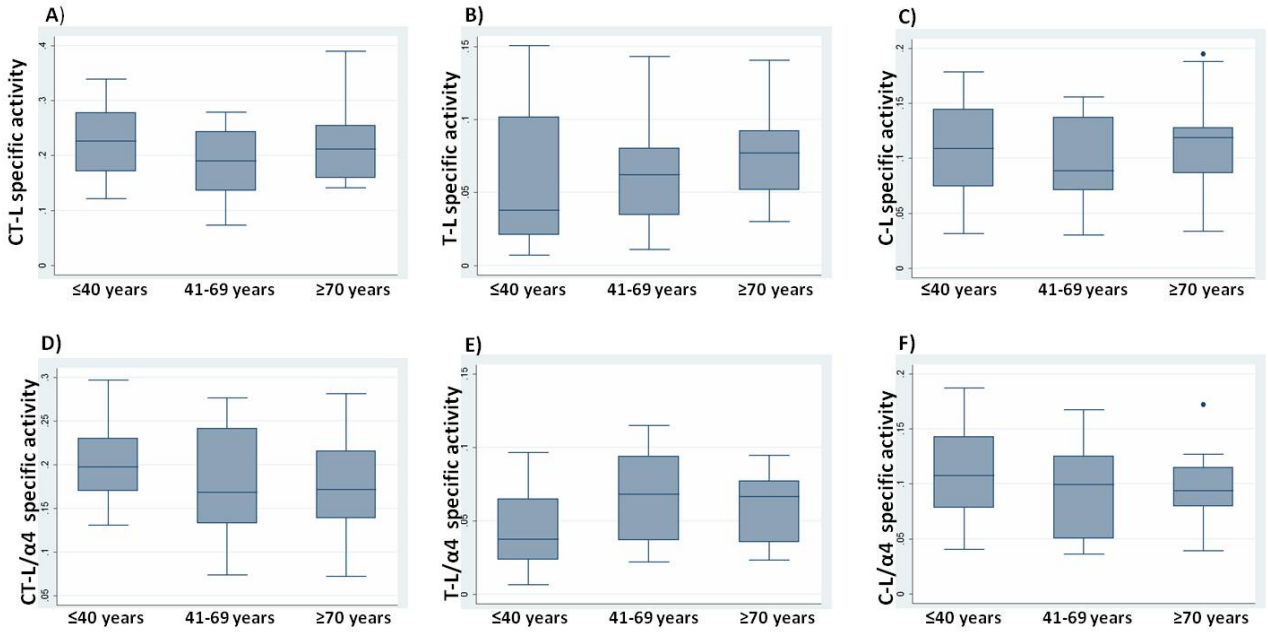


Figure 31. Proteasomes functionality in human *v. lateralis* muscle of LLMI patients. Proteasomes specific activities were analyzed in crude protein extracts from human *v. lateralis* muscle of young (≤40 years, n=12), middle-age (41-69 years, n=24) and elderly (≥70 years, n=13) LLMI patients. Hydrolysis of the fluorogenic peptides LLVY-AMC (**A**), VGR-AMC (**B**) and LLE-AMC (**C**) measured the chymotripsin-like (CT-L), the trypsin-like (T-L) and the caspase-like (C-L) specific activities, respectively. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Differences among age groups were analyzed by using ANOVA test and a *p* value<0.05 was considered statistically significant. None of the three proteasomes activities (expressed as nmol/mg*min) was affected by aging (**A**, p=0.85; **B**, p=0.37; **C**, p=0.78). Same result emerged dividing specific activities by α4 relative content : CT-L/α4, p=0.63 (**D**); T-L/α4, p=0.51 (**E**); C-L/α4, p=0.78 (**F**).

Stratifying our data by gender, in order to test whether proteasomes activities might be differently affected by aging in male and female LLMI patients. We found an age-related increase in the T-L/α4 ratio in male subjects (p=0.026) (**fig. 32B**), but not in females (p=0.66) (**fig. 32A**).

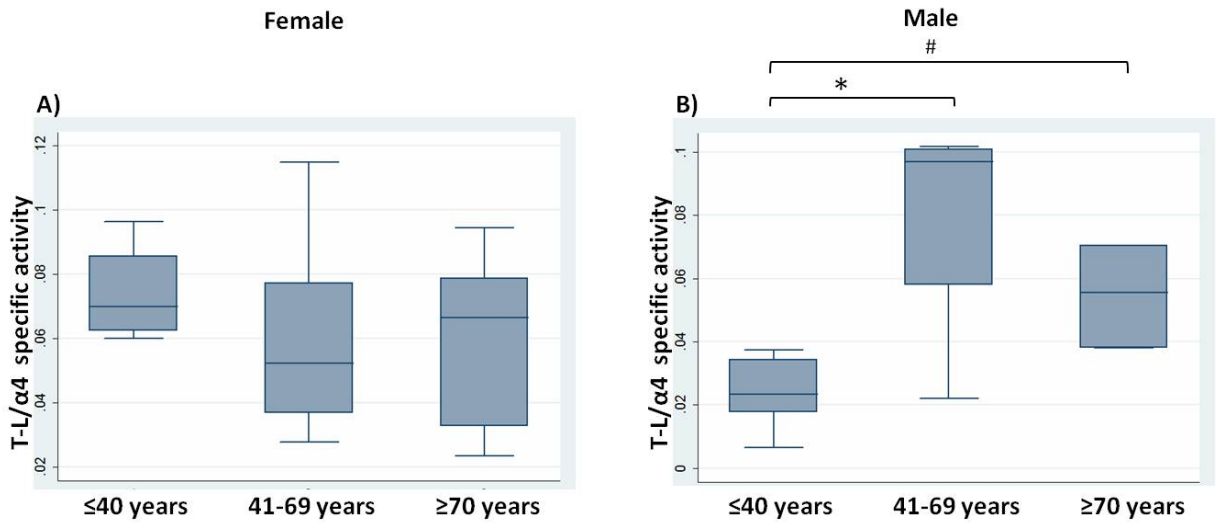


Figure 32. Proteasome trypsin-like activity/α4 relative content in human *v. lateralis* muscle of female and male LLMI patients. Proteasome trypsin-like specific activity was measured by using VGR-AMC substrate in crude protein extracts from *v. lateralis* biopsies of females (F) and males (M) belonging to young (≤40 years, F n=5, M n=7), middle-age (41-69 years, F n=11, M n=13), and elderly (≥70 years, F n=10, M n=3) LLMI patients and normalized for α4 relative content. We considered the mean of three independent experiments; in graph box data are expressed as median and respective quartiles. Differences among age groups were analyzed by using ANOVA and Student's *t* tests and a *p* value<0.05 was considered statistically significant. Only in males (**B**, $^{\bullet}$p=0.026), but not in females (**A**, p=0.66) a slight age-related increase in trypsin-like activity/α4 relative content was observed. In particular in male patients the comparisons between the young group and the middle-age one (**B**, *p=0.018), and between the young and the elderly (**B**, $^{\#}$p=0.023) were statistically significant.

4.B ANALYSIS OF PROTEASOME IN SERUM OF HEALTHY SUBJECTS AND LLMI PATIENTS

Although proteasomes are mainly linked to intracellular protein degradation, several studies have reported their presence in extracellular spaces and fluids, as intact and proteolitically active enzymes, named circulating proteasomes (c-proteasomes). The incidence and relevance of c-proteasomes have been investigated in different pathological conditions, where their levels often correlated with disease activity and clinical outcome (Sixt et al., 2008). However, at present no data are available concerning c-proteasome levels in patients with restricted mobility, as well as during healthy aging.

4.B1 CIRCULATING PROTEASOME CONCENTRATION IN HEALTHY SUBJECTS

Firstly we measured c-proteasomes levels in serum from young (≤40 years) and old healthy subjects (≥70 years), by ELISA immune-assay.

A statistically significant decrease of c-proteasomes levels in elderly subjects was observed ($p=0.0002$) (**fig. 33A**); this result also emerged by stratifying according to gender, both in females ($p=0.0001$) (**fig. 33B**) and male subjects ($p=0.014$) (**fig. 33C**).

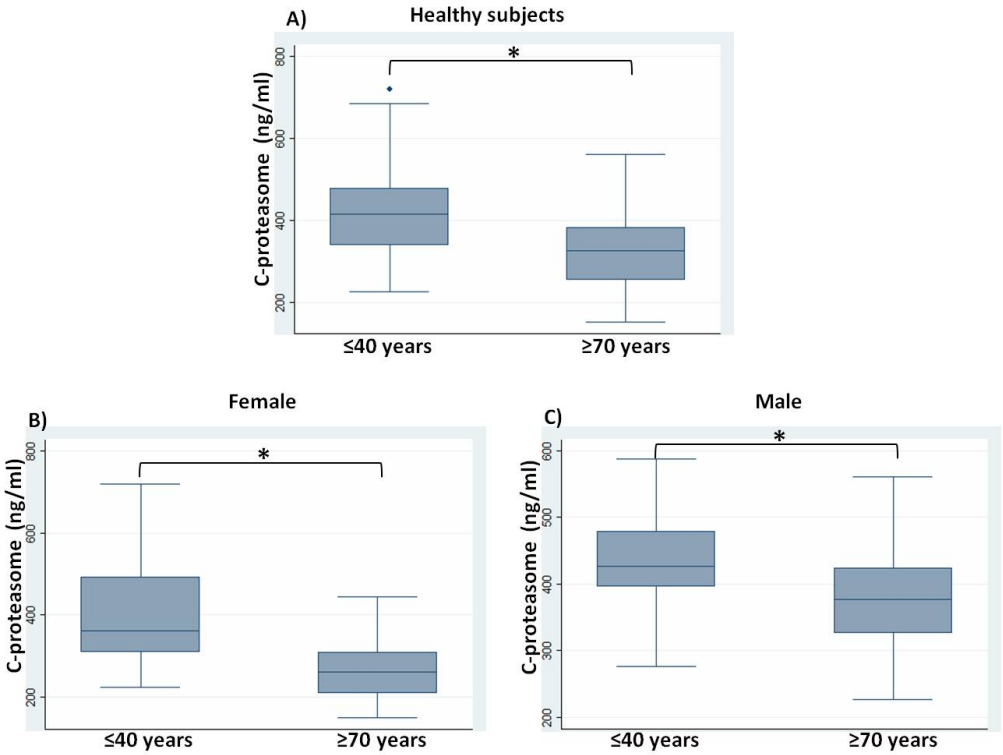


Figure 33. Serum levels of c-proteasome in healthy donors of different age. Circulating proteasomes concentration was detected by a home-made ELISA assay in serum samples from young (≤40 years, n=33) and elderly (≥70 years, n=67) healthy subjects. Data were normalized for a sample used as internal control and loaded in every plate. Comparison between the two age groups was calculated by using Student' *t*-test and a *p* value<0.05 was considered statistically significant. A decrease of c-proteasomes levels, expressed as ng/ml, was observed in elderly respect young subjects (**A**, *p=0.0002). Stratifying by gender, this result was confirmed also in old females (**B**, *p=0.0001) and in old male donors (**C**, *p=0.014).

Additionally, we tested whether gender impinges upon c-proteasomes levels, by comparing female and male donors of the same age group. From this analysis we found that c-proteasomes levels were higher in elderly male than old females (p<0.0001) (**fig. 34B**), but similar within the young group (p=0.20) (**fig. 34A**).

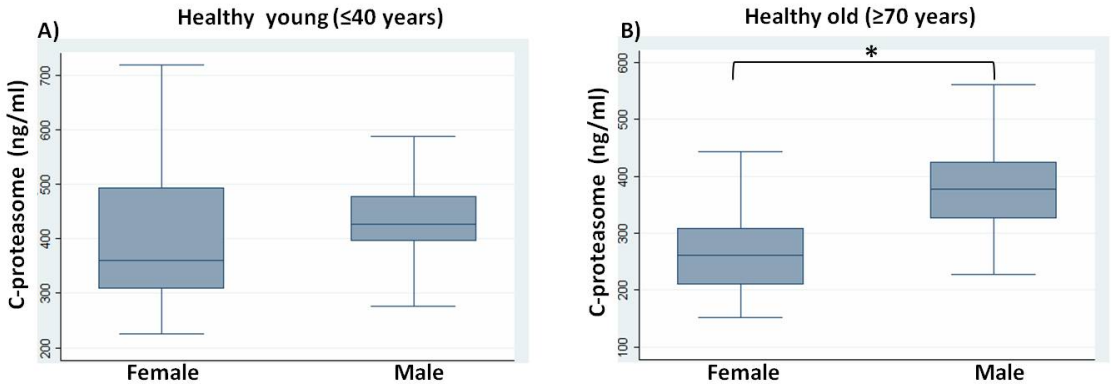


Figure 34. Serum levels of c-proteasomes in healthy male and female donors of different age. Circulating proteasomes in serum samples from young (≤40 years, F n=19, M n=14) and elderly (≥70 years, F n=31, M n=36) healthy subjects. Data were normalized for a sample used as internal control and loaded in every plate. Comparison between female and male donors of the same age group was calculated by using Mann-Whitney test and a *p* value<0.05 was considered statistically significant. The analysis showed that c-proteasomes levels were higher in elderly male subjects than old females (**B**, *p<0.001), with no gender difference within the young group (**A**, p=0.20).

4.B2 CIRCULATING PROTEASOME CONCENTRATION IN LLMI PATIENTS

Secondly, we measured c-proteasomes levels in serum of LLMI patients of different age. Remarkably also in this case, a significant decrease of c-proteasomes levels during aging was observed in whole samples size (p=0.04) (**fig. 35A**), but not when females (p=0.13) (**fig. 35B**) and male subjects (p=0.25) (**fig. 35C**) were analyzed separately.

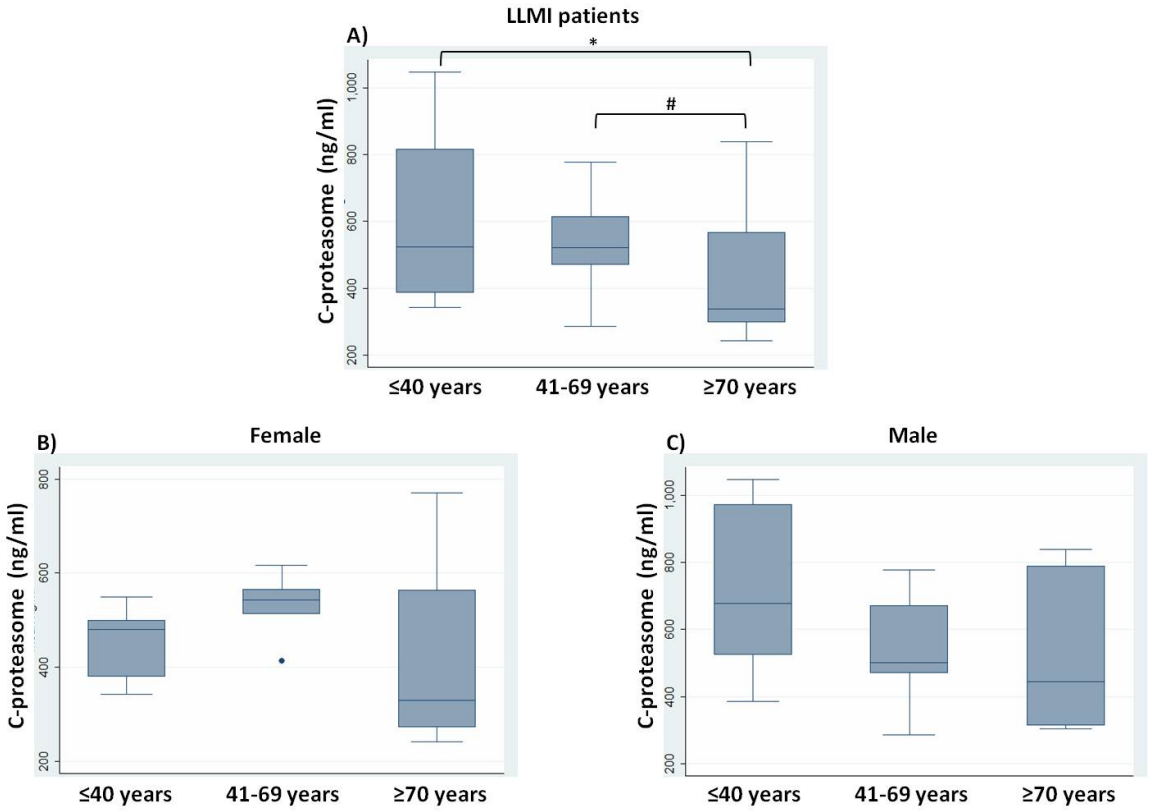


Figure 35. Serum levels of c-proteasomes in LLMI patients of different age. Circulating proteasomes concentration was measured in serum samples from young (≤40 years, n=11), middle-age (41-69 years, n=15) and elderly (≥70 years, n=20) LLMI patients. Data were normalized for a sample used as internal control and loaded in every plate. Comparison among the three age groups was performed by using Kruskal-Wallis test and a *p* value<0.05 was considered statistically significant. The analysis showed that c-proteasomes levels (ng/ml) decrease with aging (**A**, •p=0.04); in particular the comparisons between the young and the elderly (**A**, *p=0.028) and between the middle-age group and the old one (**A**, $^{\#}$p=0.045) were statistically significant. Stratifying by gender, no age-related modifications were observed in females (**B**, p=0.13) and males (**C**, p=0.25) LLMI patients.

Additionally, analyzing the three different age groups separately, in contrast to healthy subjects, no difference according to gender was found for c-proteasomes levels in LLMI patients (**fig. 36A-C**).

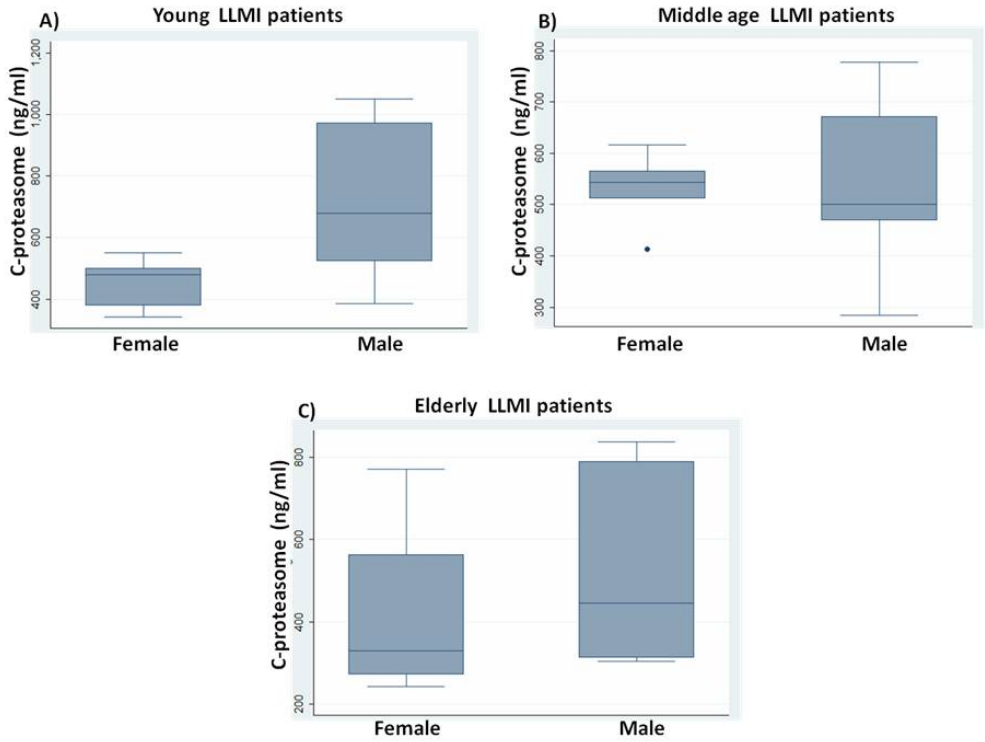


Figure 36. Serum levels of c-proteasomes in male and female LLMI patients of different age. Circulating proteasomes in serum samples from young (≤40 years, F n=5, M n=6), middle-age (41-69 years, F n=5, M n=10) and elderly (≥70 years, F n=14, M n=6) LLMI patients. Data were normalized for a sample used as internal control and loaded in every plate. Comparison between female and male patients within the same age group was performed by using Mann-Whitney test and a *p* value<0.05 was considered statistically significant. No difference according to gender was observed for all three age groups (**A**, the young group; **B**, the middle-age group; **C**, the elderly group).

4.B3 COMPARISON OF CIRCULATING PROTEASOME LEVELS BETWEEN HEALTHY SUBJECTS AND LLMI PATIENTS

Taking into account that in several pathological conditions, such as cancer, autoimmunity and inflammatory diseases, c-proteasomes levels were higher than those of healthy controls, we investigated whether this phenomenon was also present in LLMI patients. Thus we compared c-proteasomes levels between LLMI patients and healthy subjects, within the same age group.

In young subjects c-proteasomes levels were higher in LLMI patients than healthy donors (p=0.0096) (**fig. 37A**); while only a trend was observed for the same comparison between patients and healthy within the elderly group (p=0.06) (**fig. 37A**).

Stratifying by gender, in order to test whether LLMI pathology differently affected levels of c-proteasomes in male and female subjects, in the same age groups. Higher levels of c-proteasomes were observed in LLMI patients respect to healthy donors within the female elderly group (p=0.0065) (**fig. 37B**) with no modification in the female young group (p=0.19) (**fig. 37B**). On the contrary, c-proteasomes levels were higher in young male patients than in young male healthy donors (p=0.017) (**fig. 37C**), but similar levels were reported in the elderly (p=0.18) (**fig. 37C**).

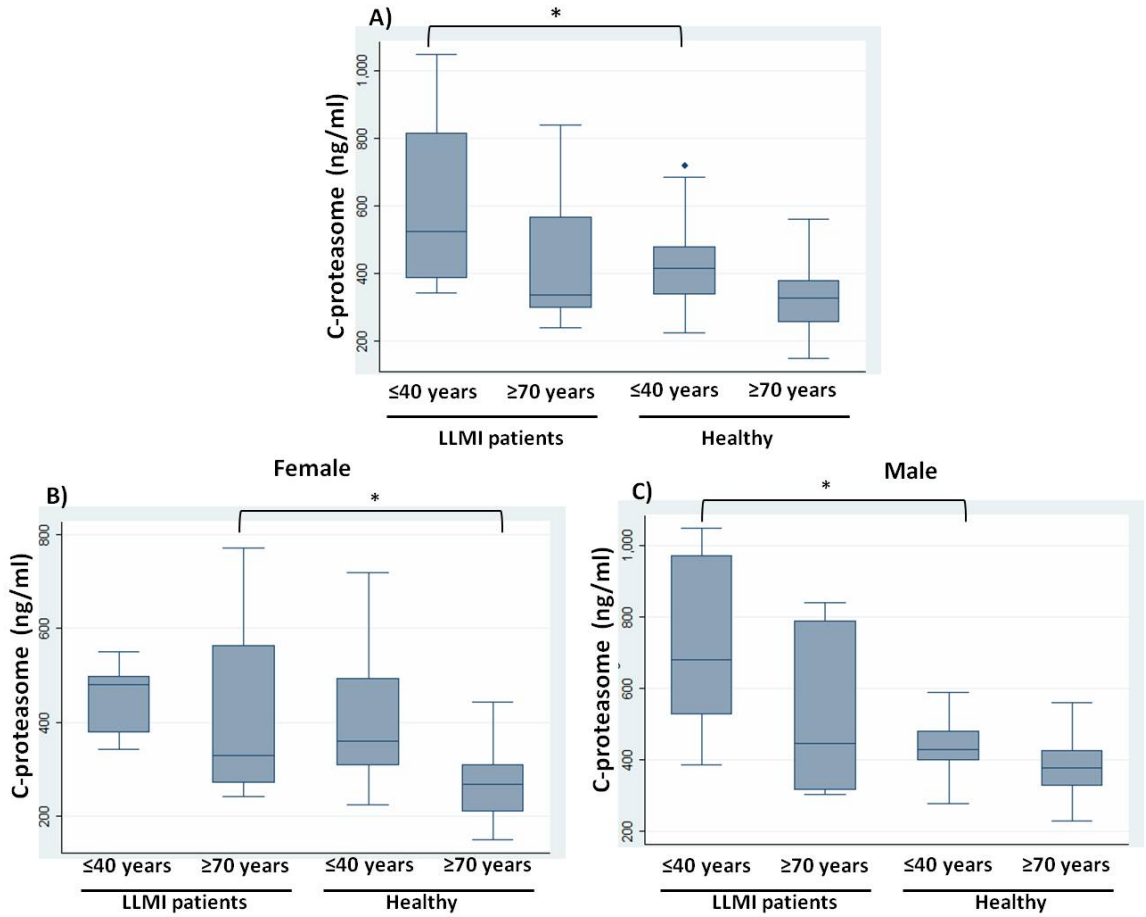


Figure 37. Comparison of c-proteasomes serum levels between healthy subjects and LLMI patients. Circulating proteasomes concentration in serum samples from young (≤40 years, healthy n=33, LLMI n=11) and elderly (≥70 years, healthy n=67, LLMI n=20) donors. Data were normalized for a sample used as internal control and loaded in every plate. Differences between groups were calculated by using Kruskal-Wallis test and a *p* value<0.05 was considered statistically significant. We observed higher c-proteasomes levels in LLMI patients than in healthy subjects for the young (**A**, *p=0.0096), whereas only a trend was observed in the old group (**A**, p=0.06). Stratifying by gender the young (≤40 years, healthy F n=19, healthy M n=14; LLMI F n=5, LLMI M= 6) and the elderly (≥70 years, healthy F n=31, healthy M n=36; LLMI F n=14, LLMI M n=6) subjects, we found significant higher levels of c-proteasomes in female patients respect to female healthy subjects within the elderly group (**B**, *p=0.0065), but similar levels in the female young donors (**B**, p=0.19). On the contrary, c-proteasomes levels were higher in young male patients respect to young male healthy subjects (**C**, *p=0.017), with no difference in the male elderly group in the comparison between patients and healthy (**C,** p=0.19).

4.B4 CORRELATION BETWEEN C-PROTEASOMES LEVEL, ANTHROPOMETRIC PARAMETERS AND CYTOKINES IN HEALTHY SUBJECTS

At last, we performed correlation analyses between c-proteasomes level and additional variables included in the MYOAGE database, described by McPhee and co-workers (McPhee et al., 2013). In particular, informations on anthropometric and body composition parameters, as well as muscle function (handgrip strength and quadriceps torque best effort) were used. Additionally, we performed the same correlation analyses between c-proteasomes level and the level of some adipokines (leptin, adiponectin and resistin) as well as IGF-1, measured in serum of healthy subjects, by co-workers in the laboratory (Bucci et al., 2013). As shown in **table 10**, in the whole sample size of healthy subjects, we observed a positive correlation between c-proteasomes level with handgrip and quadriceps strength as well as IGF-1. On the contrary, a negative correlation was found between c-proteasomes level and total fat mass, both in weight (kg) than in percentage (%), as well as for leptin, and resistin/IGF-1 ratio.

Healthy subjects		
	C	p
Handgrip (kg)	0.441	<0.0001
Quadriceps strength (N/m)	0.481	<0.0001
Total fat mass (kg)	-0.249	0.014
Total fat mass (%)	-0.389	<0.0001
Leptin (ng/ml)	-0.27	0.007
IGF-1 (ng/ml)	0.319	0.001
Resistin/IGF-1 ratio	-0.213	0.034

Table 10. Correlation analysis of c-proteasomes levels with anthropometric measurements and cytokines levels in all healthy subjects (n=99). In Table are shown positive and negative correlations between c-proteasomes concentration and different anthropometric variables, as well as cytokine levels. C=Spearman's correlation coefficient.

Stratifying by age, we observed no significant correlation between c-proteasomes level and the above described variables in the young healthy subjects. On the contrary, in the old healthy donors we found a significant positive correlation between c-proteasomes level and weight, handgrip, quadriceps strength; while total fat mass (%), leptin and leptin/adiponectin ratio correlated negatively with c-proteasomes level (**table 11**).

	Young		**Old**	
	C	**p**	**C**	**p**
Weight (kg)	**0.113**	**0.531**	**0.269**	**0.029**
Handgrip (kg)	**0.208**	**0.253**	**0.447**	**<0.0001**
Quadriceps strength (N/m)	**0.295**	**0.095**	**0.376**	**0.002**
Total fat mass (%)	**-0.113**	**0.0538**	**-0.45**	**<0.001**
Leptin (ng/ml)	**-0.091**	**0.615**	**-0.359**	**0.003**
Leptin/adiponectin ratio	-0.092	0.611	-0.293	0.017

Table 11. Correlation analysis of c-proteasomes levels with anthropometric measurements and cytokines levels in young (n=33) and elderly (n=66) healthy subjects. In Table are shown positive and negative correlations between c-proteasomes concentration and different anthropometric variables, as well as cytokines. C=Spearman's correlation coefficient.

We did not further investigated possible gender-related differences, because the number of subjects representative of two genders was unbalanced to obtain reliable results.

5. DISCUSSION

Human aging and longevity are complex and multifactorial traits, that are the result of the interaction between environmental, genetic, epigenetic and stochastic factors, each contributing to the overall phenotype (Salvioli et al., 2013; Rattan 2007; D'Aquila et al., 2013). These complex processes act differently in each organ system, tissue and even in single cells of the body, determining a different aging rate for each of them. Thus, the aged body could be considered as a mosaic of tissues and organs displaying different level of senescence, a situation we proposed to indicate as a dynamic "aging mosaic" (Cevenini et al., 2010). An example is the brain, where different regions such as cortex, hippocampus and cerebellum show different levels of neurodegeneration and inflammation (Mishto et al., 2006a).

Despite some structural changes in its morphology and a reduced regenerative capacity, human liver seems to be marginally affected by aging (Schmucker, 2005; Schmucker et al., 2011; Tajiri et al., 2013). Accordingly, orthotopic liver transplants where the age of the donor is considerably higher than that of the recipient, have notably increased in the last years, and liver graft from aged subjects has, in specific conditions, function and duration comparable to those achievable with liver grafts from young donors (Cescon et al., 2003; Gastaca et al., 2005; Cescon et al., 2008; Schmeding et al., 2010). This evidence seems to indicate for the liver a peculiar aging pattern where the biological age does not correspond to the chronological age of the organ/donor, and indirectly confirms its capacity to rejuvenate when in presence of the appropriate microenvironment (Conboy et al., 2005).

On the contrary, a decrease in mass, strength and power in human skeletal muscle (a phenomenon defined as sarcopenia) occurs during human aging, leading to a progressive loss of mobility and increased risk of falls and invalidity. Different factors are involved in the onset of skeletal muscle wasting, including denervation, inactivity, hormonal and nutritional changes, inflammation and imbalance between protein synthesis and degradation, among others (Lang et al., 2010; Sipilä et al., 2013). In fact, alterations in proteolysis may contribute to the onset and progression of several age-related pathological conditions, including skeletal muscle wasting and sarcopenia (Ciechanover, 2013), as well as to the aging phenotypes (López-Otín et al., 2013).

The ubiquitin-proteasome system (UPS) is one of the most important cellular pathways for intracellular degradation of short-lived as well as damaged proteins, thus contributing to the maintenance of cellular homeostasis (Chondrogianni et al., 2010; Koga et al., 2011). Several studies reported that the content and the activity of proteasomes, the catalytic core of UPS, are variably affected by aging in different cell types and organs, both in human and animal models (Baraibar et al., 2012). To date, studies of liver proteasomes composition and functionality during aging produced controversial results in rodents (Baraibar et al., 2012) and no data are available on the age-related modifications of proteasomes in human liver. However, our previous observations on proteasome dynamic in normal fetal and adult human livers demonstrated that i-proteasome is absent in hepatocytes during fetal life, but it is detectable at cytoplasmic level from the third month of extrauterine life (Vasuri et al., 2010).

Regarding skeletal muscle, most of the studies present in the literature have been performed in animal models and they were mainly focused on ubiquitin pathway, rather than on proteasomes (Vinciguerra et al., 2010). In rat, an age-related increase of constitutive proteasomes and i-proteasomes content in type I and II fibers of skeletal muscle (Husom et al., 2004; Ferrington et al., 2005), as well as in gastrocnemius and soleus muscles of aged sarcopenic animals (Altun et al., 2010) has been reported. However, despite the age-related induction of the i-proteasome, this isoform seems to represent a minor fraction of total proteasomes in rat skeletal muscle (Dahlmann et al., 2001). Only one study examined proteasomes functions in human *rectus abdominis* muscle and no age-related changes emerged (Bossola et al., 2008).

On these premises, we aimed at analyzing **proteasomes changes in human liver and skeletal muscle during aging**, taking advantage of two research projects in which our group was involved: the Italian Ministry of University and Research Grant PRIN2008 ''Pretransplant liver biological age and age-mismatch between donor and recipient as new predictors of transplant outcome'' to Dr. Gianluca Grazi, Regina Elena Hospital, Rome, and the FP7 European project "MYOAGE - Understanding and Combating Age-related Muscle weakness", coordinator Prof. Gillian Butler-Browne, INSERM, Paris, France. Additionally, we studied for the first time the levels of circulating proteasomes, the extracellular proteasome isoforms, in sera of young and aged healthy subjects as well as patients with lower limb mobility impairment (LLMI).

5.1 LIVER STUDY

The analyses of **proteasomes composition** at protein level showed that i-proteasome catalytic subunits, concomitantly to their homologous of constitutive proteasome, were expressed in different cell types of human liver, including hepatocytes, and incorporated into active 20S proteasomes. The histological analysis of these liver biopsies showed no signs of necrotic damage or morphologically evident inflammation; thus the presence of i-proteasomes even in young subjects suggests that, at variance with other organs such as brain, in liver a basal level of pro-inflammatory cytokines is warranted, probably sustained by the continuous exposure to antigenic stimuli coming from the gut microbiota via the portal vein. In fact hepatocytes express a number of pattern recognition receptors (PRR), such as TLR3, RIG-I and MDA5 that can trigger the production of inflammatory cytokines (Broering et al., 2011). At the same time a permanent activation of intracellular signaling pathways could be also possible (Ebstein et al., 2012; Grimm et al., 2012) although such notions remain to be formally addressed.

As far as age-related changes of **proteasomes content**, we detected an age-related decrease of β1 and a non-monotonic variation of β2 catalytic constitutive subunits. By stratifying the sample size according to gender, the β1 subunit decreased with aging only in male donors, while an increase in β5i/α4 ratio in the whole sample set and both in female and male subjects was observed. This suggests that during aging a shift toward proteasomes containing immunosubunits occurs, as recently described also in aged rat liver (Gohlke et al., 2014). Concomitantly, PA28α subunit representative of PA28αβ proteasome regulator decreased with age in whole sample size and in male elderly biopsies. Interestingly, IHC analyses suggested that this age-related modifications occurred mainly in the cytosol of hepatocytes. As no variation in the amount of PA700 regulator occurred, it is possible that liver aging is accomplished by a variation in the amount of hybrid PA700-20S-PA28 and PA28-20S-PA28 proteasomes. Taking into account that intermediate type proteasomes as well hybrid proteasomes might have different substrate preferences and increase antigenic diversity (Gohlke et al., 2014; Vigneron et al., 2012), further investigations on changes in proteome repertoire during liver aging should be addressed. Moreover, at present, no data are available on gender contribution to proteasomes content, and considering that biopsies from young female donors were not available, additional studies on this issue should be performed.

Regarding the analysis of **proteasomes functionality**, the major finding of our study in human liver is that we did not observe any accumulation of oxidized proteins, a classical hallmark of normal aging and age-related diseases. At the same time, we did not observe any age-related changes in the amount of poly-ubiquitin conjugates, including those on Lys48, which represents the main signal for proteasome degradation, suggestive of a preserved functionality. Indeed, only a drop of the T-like and C-like activities normalized for proteasomes content were observed in male donors of middle-age group, with not statistically significant differences between the young and the old groups. Taking into account that our investigations were performed on crude protein extracts of liver biopsies, we were not able to discriminate between 20S and 26S proteasomes activity, as well as to assess the role of others possible mechanisms of regulation. Thus, further tests on purified proteasomes are needed to assess whether aging might impinge upon these two isoforms at different extent. Additionally, our data also suggest that gender could affect proteasomes functionality and oxidative stress response, thus this issue has to be considered when performing studies in humans (Baumgartner et al., 1999; Fanò et al., 2001).

5.2 MUSCLE STUDY

The analyses of **proteasomes composition** showed at the level of gene expression an age-related increase of the inducible components, such as β1i and β5i catalytic subunits, and PA28β subunit of PA28αβ regulatory complex, in healthy subjects, along with no change of constitutive components, represented by α6 and β1. The same trend, even if not significant, was also detected in LLMI patients, suggesting that the expression of inducible components of proteasome can be up-regulated during physiological aging as well as physical inactivity, likely due to the presence of pro-inflammatory cytokines, such as TNF-α that may activate the NF-kB pathway, as recently demonstrated (Barberi et al., 2013). However, we observed that i-proteasomes were present at very low protein levels in human *v.lateralis* skeletal muscle. In particular, β1i and PA28β subunits were barely detectable in biopsies of LLMI patients and all the three inducible components, including β5i, were absent in both young and elder healthy donors. These data were also confirmed by IHC in LLMI subjects; indeed, in the rhabdomyocytes, the staining for the constitutive component of proteasomes, represented by β1, was strong at cytosolic level, while in the same

cell type the signals corresponding to β1i and β5i were absent or barely detectable. Therefore, it is to suppose that post-transcriptional and/or post-translational mechanisms may impinge upon protein synthesis, assembly and maturation of inducible proteasomes, in terminally differentiated rhabdomyocytes. The same phenomenon has been observed in the brain of lymphocytic choriomeningitis virus-infected mice (Kremer et al., 2010), where an accumulation of precursors but not mature i-proteasomes was shown in microglia cells. Thus, it is possible that in rhabdomyocytes, despite be present at transcriptional level, the presence of mature 20S i-proteasome might be deleterious or not necessary for muscle cell metabolism. Moreover, as Altun and co-workers showed, immunoblotting analysis performed by using antibody directed against i-proteasome subunits in crude protein extracts has low sensitivity, thus a more sensitive assay involving specific probes for active β subunits (Kessler et al., 2001) may help to further investigate the contribution of i-proteasome in human skeletal muscle cells.

Remarkably, a different scenario was observed when we studied myoblast cell cultures obtained from *v. lateralis* muscle biopsies of young and old donors and propagated *in vitro*. Indeed, by performing western blotting analysis on crude protein extracts of myoblast cell cultures from young and elderly subjects, we found that these muscle precursors expressed high level of inducible components (β1i, β5i, PA28β), concomitantly with constitutive proteasomes. Our findings thus indirectly provide new evidence supporting the notion that i-proteasome may be required for the maintenance of proliferative capacity of muscle cells. Indeed, an enhanced expression of genes encoding subunits of the 20S i-proteasome in human embryonic stem cells coupled to their down-regulation as the cells progress into differentiation has been reported, suggesting a role in the degradation of cell cycle regulatory components and/or differentiation inducing factors (Atkinson et al., 2012). Furthermore, an involvement of i-proteasome in the protection against oxidative stress, through its ability to remove more efficiently than constitutive proteasomes, oxidized and damaged proteins, has been observed (Seifert et al., 2010; Pickering et al., 2012a). This propriety seems to be particularly required in embryonic stem cells where these proteins are present at high levels and their elimination is necessary for normal embryonic development *in vivo* (Hernebring et al., 2006; Hernebring et al., 2013). Finally, Ziyou and co-workers reported for the first time that i-proteasomes, independently of constitutive proteasomes, play an important role in proper skeletal muscle development. Indeed, down-regulation of i-proteasomes increased oxidized protein levels and induced apoptosis, resulting an impaired C2C12 skeletal muscle differentiation (Cui et al., 2014). We also observed that the

content of inducible components in myoblast was not affected by aging, as well as culture conditions (e.g. different oxygen tension). This fact is in agreement with recent findings showing that muscle progenitors from young and old donors display similar characteristic *in vitro*, such as proliferative, differentiation and senescent profile (Asharidah et al., 2013), and the reduced regenerative capacity of stem cells during aging may be related to an hostile microenvironment (Lepperdinger et al., 2013; Barberi et al., 2013), probably arising from systemic chronic inflammation which is typical of elderly subjects (Franceschi et al., 2007). It is however to note that the myoblasts that we could analyze were at quite advanced culture passage, so that all these results should be further confirmed with more early-passages cultures.

As far as **proteasome content,** in biopsies from healthy subjects we found an age-related increase in β5 protein level and in the β5/α4 ratio, suggesting an increased content of constitutive proteasomes, as also reported in animal models (Husom et al., 2004; Ferrington et al., 2005; Altun et al 2010), which could be mediated by an enhanced subunit translation, more efficient assembly or slower degradation of proteasomes during aging. However, as the amount of muscle biopsies from young and elderly healthy subjects was not sufficient to measure proteasomes functionality, further investigations were not possible. It would be of interest to assess whether this increased proteasomes content is paralleled by an enhanced proteolytic activity (thus the proteasomes activity per unit of proteasomes would be maintained) or it represents a compensatory mechanism to counteract the age-related decline in proteasomes activity during aging, as reported in several studies in human and rodents (Baraibar et al., 2012). Moreover, considering that the healthy donors were only females, further investigations are needed to assess whether males undergo the same age-related changes (Baumgartner et al., 1999). Of note, by comparing healthy subjects and LLMI patients, independently of their age, we observed, in the latter group, a marked increase of constitutive proteasomes content, possibly contributing to muscle wasting occurring during physical inactivity. A similar increase of proteasomes content has been described in other pathological situations, such as neurodegeneration (Mishto et al., 2006a; Bellavista et al., 2013). Moreover, in physically inactive patients, at variance with what observed in healthy subjects, no age-related differences in proteasomes content were found when the young and the old groups were compared. In fact, only a non-monotonic variation in β5/α4 ratio, in whole sample size and in female donors, was observed.

Regarding **proteasome functionality**, it appears preserved during aging in female LLMI patients, and only a slight increase in the trypsin like activity/α4 relative content in male subjects emerged. This latter phenomenon might represent a compensatory mechanism to counteract the increased levels of oxidized proteins found in male, but not in female, LLMI patients. On the contrary, no age-related modifications of poly-ubiquitinylated protein content were observed, nor in male nor in female. This fact suggests a maintained balance between ubiquitynilation and protein degradation by 26S proteasomes.

5.3 CIRCULATING PROTEASOMES STUDY

A third part of my study focused on the detection of **circulating proteasomes (c-proteasomes) in sera of healthy subjects as well as LLMI patients** recruited in the framework of the MYOAGE European project. We also correlated circulating-proteasomes levels with anthropometric measurements and parameters of body composition, muscle function as well as cytokines.

Although proteasomes are mainly linked to intracellular protein degradation, several studies have reported their presence in extracellular spaces and fluids, as intact and proteolytically active enzymes. The incidence and relevance of c-proteasomes have been investigated in different pathological conditions, where their levels often correlated with disease activity and clinical outcome (Sixt et al., 2008). However, at present, no data are available concerning possible correlation between c-proteasomes levels and muscle function during aging.

We found for the first time that, in healthy subjects, levels of c-proteasomes decreased in elderly subjects (≥70 years) and this phenomenon was present in both men and women. Secondly, we observed that c-proteasomes levels were higher in elderly male than females, thus suggesting that gender-related factors, such as hormones, might impinge upon c-proteasomes levels only in the late adulthood. In LLMI patients c-proteasomes levels followed the same trend, as a decrease was observed in the group of elderly patients, with not clear gender difference. This fact might be related to the number of subjects analyzed as well as to the variability among LLMI patients.

Additionally, gender of donor does not seem to affect c-proteasome levels in the three age groups considered separately, suggesting that LLMI condition might mask the gender issue.

Moreover, we found that LLMI patients had an increased levels of c-proteasomes compared to healthy donors. Similarly, increased levels of c-proteasomes have been observed in some pathologies, such as malignancy, autoimmune diseases, trauma and sepsis (Sixt et al., 2008). Specifically this effect emerged within the young group, in all the samples and in male LLMI patients; on the contrary in female patients the difference emerged in the elderly group. The discrepancy might be in part related to the gender contribution we found in healthy subjects. At present, the origin and the role of c-proteasomes in blood have not been fully elucidated. Recent findings suggested that proteasomes might be released by active mechanisms, such those observed in choroid plexus cells (Muller et al., 2012) and in mice mesenchimal stem cells (Lai et al. 2012). The latter study showed that both constitutive and inducible proteasomes subunits were present in exosomes vesicles and might have a potential therapeutic effect in a mouse model of myocardial infarction by degrading oligomers (Lai et al., 2012). Correlation analysis in healthy subjects showed that levels of c-proteasomes were positively correlated to muscle strength and negatively to total fat mass, in the whole sample size and in particular in the elderly subjects. Further investigations are needed to assess mechanisms underlying these relationships, however the significant positive association between c-proteasomes and muscle strength suggests an important role of c-proteasomes in the maintenance of muscle function.

6. CONCLUSIONS

This study was aimed at identifying possible age-related modifications of proteasome composition, content and functionality of two different human tissues such as liver and skeletal muscle. To our knowledge, this is the first time that such an ambitious task is tackled in human aging studies. We could reveal that:

i) proteasomes composition and functionality does not seem to be affected by aging in human liver and skeletal muscle, except minor changes/remodeling occurring especially in the middle-age subjects

ii) while i-proteasomes are induced in liver as soon as the third month of extrauterine life, they play a marginal role in the aging of skeletal muscle, both in healthy condition and in LLMI patients, suggesting that these two tissues might have different request in term of proteasomes composition and substrate specificity

iii) further investigations are needed to elucidate the contribution of i-proteasomes to cell proliferation and differentiation of muscle cell precursors

iv) as in LLMI patients we observed an increase of constitutive proteasomes content, additional studies should be performed to understand if this phenomenon is the cause or the consequence of LLMI pathological condition

v) some proteasomes-related parameters seem to be affected by gender , which is an important issue to take into account when performing studies in humans

vi) we measured c-proteasomes levels in serum of subjects participating to the MYOAGE study and we could observe that such levels decrease during aging, in both healthy subjects and LLMI patients, but their function and origin have yet to be fully understood

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