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Ciclo XXXIV

Evaluation of Galectin-3 in the muscle-to-bone crosstalk

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Summary

Abstract (Italiano)

La Galectina-3 (Gal-3) è una lectina pleiotropica che interagisce con diverse proteine intracellulari ed extracellulari influenzando numerosi processi biologici ed è coinvolta in diverse condizioni fisiologiche e patofisiologiche. Nonostante il tema della Gal-3 sia stato molto studiato, alcuni meccanismi d'azione rimangono ancora non completamente compresi tra cui i meccanismi molecolari nello spazio intra ed extracellulare e la relazione tra questi meccanismi, nonché il ciclo regolatorio ad essi associato.

Poiché è ben descritto che la Gal-3 è ampiamente espressa sia nell'osso in via di sviluppo che in quello maturo e che la sua espressione è sotto il controllo del regolatore principale della crescita ossea RUNX2, ciò potrebbe suggerire che Gal-3 possa essere un attore chiave in tutte le fasi del metabolismo osseo. Inoltre, molti tipi di patologie ossee sono sorprendentemente correlati a gravi danni che colpiscono i tessuti associati all'osso. In particolare, è ben noto che ossa e muscoli sono stati recentemente identificati come organi endocrini, che secernono citochine e chemochine, attraverso le quali interagiscono per promuovere la formazione, la riparazione e il mantenimento dell'osso nel cross talk muscolo-scheletrico. Per questo, l'obiettivo generale del nostro studio è stato quello di definire il ruolo di Gal-3 nel tessuto osseo e il suo possibile coinvolgimento nel cross-talk muscolo scheletrico. In primo luogo, abbiamo riscontrato un aumento dell'espressione di Gal-3 durante il differenziamento miogenico e verificata la sua secrezione nel terreno di coltura. Successivamente abbiamo studiato l'effetto della Gal-3 ricombinante, che imita la Gal-3 extracellulare secreta dalle cellule muscolari, durante la differenziazione degli osteoblasti, che risulta nell'inibizione dell'osteogenesi. Al fine di identificare le principali classi di proteine modulate da Gal-3, abbiamo analizzato l'intero proteoma di osteoblasti differenziati in presenza della Gal-3 ricombinante, mediante spettrometria di massa. Successivamente abbiamo valutato i meccanismi di segnalazione a valle della stimolazione extracellulare della Gal-3, con un approccio protein array basato sia su proteine fosforilate che su molecole metaboliche coinvolte nel metabolismo osseo. Inoltre, attraverso un ECM Adhesion Array, abbiamo monitorato l'impatto della Gal-3 sulla capacità degli osteoblasti di interagire con le proteine della matrice extracellulare, valutando integrine specifiche della superficie cellulare e molecole di adesione. Presi insieme, i nostri risultati mostrano che la Gal-3 extracellulare regola negativamente la rigenerazione ossea inibendo vie di segnalazione come AKT / mTor pathway o la segnalazione mediata dalle BMP. La galectina-3 potrebbe essere un possibile bersaglio terapeutico per il trattamento di patologie muscolo-scheletriche, soprattutto in quelle condizioni patologiche (es. invecchiamento e infiammazione) in cui è maggiormente secreta, influenzando negativamente il metabolismo osseo.

Abstract (English)

Galectin-3 (Gal-3) is a pleiotropic lectin that interacts with different intracellular and extracellular proteins influencing numerous physiological and pathophysiological processes. Although the topic of Gal-3 has been extensively studied, it is still not completely elucidated, particularly with regard to the molecular mechanisms both in the intra- and extracellular space, and their relationship, as well as the associated regulatory cycle. Since it is well described that Gal-3 is widely distributed both in the developing and mature bone and that its expression is under control of the master regulator of bone growth RUNX2, it could suggest that Gal-3 may be a key player in all stages of bone metabolism. Many types of bone pathologies are strikingly related to severe damage inputs affecting the bone-associated tissues. In particular, it is well recognized that bone and muscle are endocrine organs, that secrete cytokines and chemokines, through which they interact with each other to promote bone formation, repair and maintenance in the bone-muscle cross talk. Therefore, the general aim of our study was to define the role of muscle-derived Gal-3 on bone tissue and its possible involvement in skeletal muscle crosstalk. First, we found an increase of Gal- 3 expression during myogenic differentiation and assessed its presence in the culture medium. Next, we investigated the effect of recombinant Gal-3, which mimics the extracellular Gal-3 secreted by muscle cells, during osteoblast differentiation, resulting in inhibition of osteogenesis. In order to identify the main classes of proteins modulated by Gal-3, we analyzed the entire proteome of differentiated osteoblasts in presence of recombinant Gal-3, through Mass Spectrometry. Afterwards we evaluate the signaling mechanisms downstream the extracellular Gal-3 stimulation, either with a protein array approach based on phosphorylated proteins or metabolic molecules involved in bone metabolism. Moreover, through an ECM Adhesion Array kit, we monitored the impact of Gal-3 on the ability of osteoblasts to interact with extracellular matrix proteins, evaluating specific cell surface proteins such as Integrins and Adhesion molecules. Taken together, our findings show that extracellular Gal-3 negatively regulates bone regeneration inhibiting fundamental pathways such as AKT / mTor pathway or BMPs signaling. Galectin-3 could be a possible therapeutic target for the treatment of muscle-skeletal diseases, especially in those pathological conditions (i.e. aging and inflammation) where there is a greater secretion of Gal-3 which negatively affects bone metabolism.

1. Introduction

1.1.BONE TISSUE

1.1.1. General features

Bone is a specialized connective tissue characterized by a considerable hardness and resistance; it consists of an **organic portion** and an **inorganic portion**.

The **organic portion** is constituted of specialized cell types (Mesenchymal Stem Cell, Osteoblast, Osteoclast and Osteocytes) and an abundant mineralized extracellular matrix (ECM) produced by these cells. The ECM is a complex mixture of proteins and glycosaminoglycans (a class of negatively charged polysaccharides). It is composed of three categories of materials:

1. Fibrous proteins that provide tensile strength (collagens, elastin etc)
2. Adhesive glycoproteins (laminin, fibronectin, tenascin etc)
3. Glycosaminoglycans and their proteoglycans that resist compressive forces

Collagens are the major structural component of the ECM[1]. Collagens provide scaffolding for the attachment of laminin, proteoglycans and cell surface receptors[1]. Twenty-eight types of collagens (I–XXVIII) have been identified so far in vertebrates[1]. Collagens are triple helical proteins that are formed from either homotrimers or heterotrimers of polypeptide chains, referred to as α-chains. α-chains have a three amino acid repeat of Gly-X-Y, where X is typically proline and Y is 4-hydroxyproline (post-translationally modified proline)[2,]

The remaining organic portion is occupied by proteins defined as Non-Collagenic Proteins, which include:

- Glycoproteins: Fibronectin (FN), produced by osteoblasts; it regulates the initial deposition of collagen fibrils[1] and maintains the integrity of the collagenous matrix[2]; Tenascin (TN-C), expressed mainly during embryogenesis or in adult tissues is restricted to sites of active tissue remodeling. TN-C acts by binding with high affinity to specific integrins such as integrin α9 or to other matrix proteins such as FN[3].Elastin, produced by a number of different cell types including fibroblasts and endothelial cells, smooth muscle cells. The elastic fibers are able to stretch many times their length, and snap back to their original length when relaxed without loss of energy.

- Osteonectin (produced by osteoblasts; it has a strong affinity to Col I and to the mineral portion and acts as a specific bone nucleator for mineralization);

- Laminin: is synthesized mainly from Mesenchymal Stem Cells and together with other extracellular matrix proteins, such as collagen IV, form a dense molecular network called basement membrane (BM), to contact with the cellular surface, having a critical for regulating cellular function[4]

- SIBLINGS proteins: Bone Sialoprotein (BSP), Osteopontin (OPN), Dentin Matrix Protein (DMP), Dentin Sialophosphoproteins (DSPP) and ECM phosphoglycoproteins (MEPE). They are characterized by the presence of an RGD (Arg-Gly-Asp) cell adhesion sequence and by an extensive post-translational modification that includes both O and N oligosaccharides, many of which are rich in sialic acid [5,6].

The **inorganic bone matrix** represents 99% of the body's deposit of Ca^{2+}, 85% of the phosphorus and 40-60% of the magnesium and sodium. It is mainly in the form of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and has the function of providing bone strength, stiffness and resistance to compressive forces[7].

1.1.2. Physiological Function

Bone is a highly vascularized dynamic tissue capable of repairing and remodeling without leaving scars[8].

Bones perform numerous vital functions:

- *Support*: they provide structural support to the whole body and cooperate with the muscles to produce precise and controlled movements [8,9].
- *Lever system*: they work like levers, and they can change the amplitude and direction of the forces generated by the skeletal muscles. The movements produced vary from the delicate movement of the fingers to the powerful changes in the position of the whole body (Martini et al. 2012).
- *Protection*: they protect many delicate organs (ribs, skull, vertebrae and pelvis respectively protect heart and lungs, brain, spinal cord and organs of the digestive and genital tract) (Martini et al. 2012).
- *Endocrine system*: they perform an endocrine function by secreting specific bone proteins such as sclerostin (SOST) which inhibits, through paracrine action, the differentiation of osteoblasts; the fibroblast growth factor-23 (FGF-23) which regulates 1-α-hydroxylase,

phosphorus and parathyroid hormone (PTH) in the blood stream; osteocalcin (OC), produced by osteoblasts, regulates glucose metabolism by acting on the insulin signaling pathway in osteoblasts and induces testosterone secretion in leydig cells of the testes by acting on a G protein coupled receptor (GPCR6A) expressed on the cell surface[10].

- *Erythropoiesis*: numerous long bones house the bone marrow in which bone marrow erythropoiesis occurs, a process through which red blood cells, white blood cells and platelets originate[11].

1.1.3. Bone Tissue Cells

Several specific cell populations reside in the bone: progenitor cells, osteoblasts, osteocytes and osteoclasts, as described in Fig. 1.

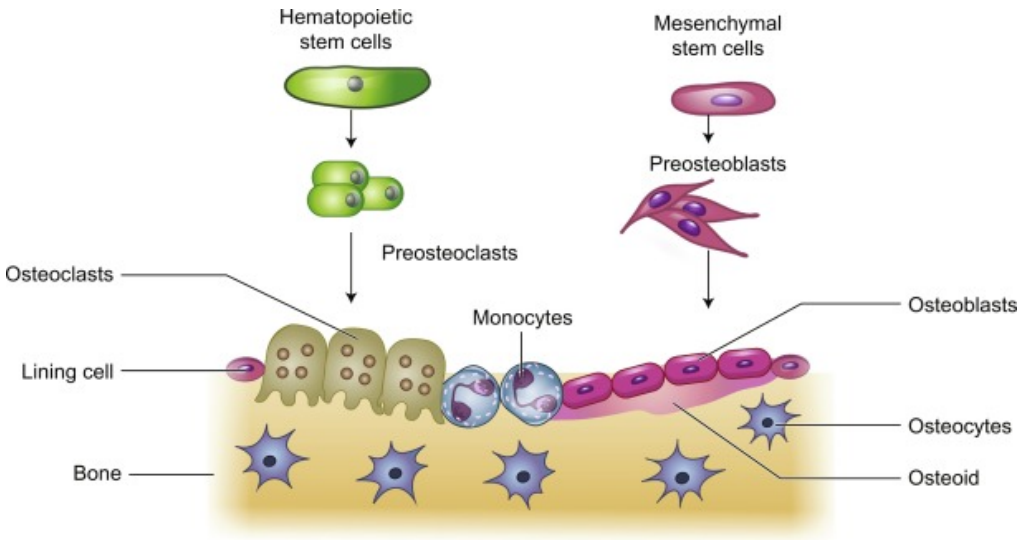


Fig.1 *– Bone tissue cells*

- *Osteoprogenitor cells, osteoblasts, osteocytes*

Osteoprogenitor cells (MSCs) have mesenchymal origin and have a fibroblastic morphology. They are fundamental cells for bone physiology, they are cells that can divide to generate daughter cells that can differentiate from the most primitive state to osteoblasts and osteocytes [12].

Osteoblasts spatially cover the internal surface of the bone and they are the cells responsible for bone formation. They work as a team to secrete and subsequently mineralize a portion of the new bone matrix[13], increase the volume of the bone and increase the trabecular thickness[14,15].

The process by which they are generated is called osteoblastogenesis: the first phase, in which a MSC is committed to become an osteoprogenitor is driven mainly by the bone morphogenic proteins (BMPs) and WNTs, while the TWIST proteins and the inhibitor of DNA binding (ID) maintain proliferation. Subsequently, the MSCs stop proliferating and begin to differentiate into osteoblasts, express proteins necessary for bone formation such as RUNX2, alkaline phosphatase (ALP), OC, Osteonectin and they start to secrete Col I and non-collagenic matrix proteins[16]. Osteoblasts can be divided into two structurally and functionally distinct subpopulations: bone lining cells which are relatively inactive, flattened and tapered cells and stretch over the bone surface to form a protective layer; active osteoblasts which are cuboidal cells, secreting cells, are characterized by the presence of a rough endoplasmic reticulum and a very extensive Golgi apparatus and they are positive for the expression of genes associated with bone formation [13,17].

Osteocytes are the mature cells of the bone and they are located within the lacunae. They have the shape of a triaxal ellipsoid, with numerous long and branched arborization that come in contact with arborization of other osteocytes through gap junctions, running inside canaliculi that radiate from each lacuna and that allow the connection between adjacent osteocytes. They derive from the differentiation of osteoblasts and are involved in the perception of both the mechanical load (contributing to skeletal homeostasis) and the metabolic needs of the skeleton (collaborating in mineral homeostasis). They are also coordinators of bone remodeling through the regulation of the activity of both osteoblasts and osteoclasts[18] and they act as endocrine cells, through the excretion of soluble factors (such as sclerostin, an inhibitor of bone development) with targets not only the bone but also distant organs, such as kidneys, muscles, and other tissues [19].

- *Hematopoietic stem cells and osteoclasts*

Osteoclasts are cells whose main task is to reabsorb bone. They are very complex cells, they have a short life, they are very active and they are the only cells in the body capable of degrading and removing a large amount of bone, despite being few. Osteoclasts develop from a process called osteoclastogenesis from the fusion of mononuclear myeloid precursors, which in turn are generated by hematopoietic stem cells (HSCs). During differentiation the precursors acquire the Colony Stimulating Factor receptor (C-FMS) which is activated by binding to their ligand, the Macrophage Colony Stimulating Factor (M-CSF) and activates proliferation. Terminal differentiation towards the osteoclastic line occurs following the expression of C-Fos and the activating receptor of the nuclear factor kB (RANK) and the

interaction of the latter with its ligand RANKL, recognized as a determining factor for the osteoclastogenesis. The mechanism of action of RANKL is based on the activation of MAPK, JNK, p38, ERK and NFkβ, on the activation of TRAF6, NOX1, RAC1 which induce a rapid and transient increase in ROS, essential for osteoclastogenic differentiation[20,21]. Mature osteoclasts are large, multinucleated cells located on the surface of the trabecular and endosteal cortical bone, often in a resorption gap created by themselves[16,22].

1.1.4. Bone turnover regulation

Bone life is characterized by two phases: **bone modeling** and **remodeling**.
The modeling takes place during the growth of the organism until it reaches maturity, it consists of two phases:

- The deposition of new bone mass by osteoblasts to increase the size of the bone and modify the bone shape according to both genetic factors and in response to load conditions.
- The bone resorption by osteoclasts (without subsequent bone formation) to create the medullary cavity and establish the cortical and trabecular architecture of the bone.

Bone remodeling occurs in adulthood to perform some important bone functions. In addition, this process also allows for the repair of minor damage to the bones subjected to loads. It is estimated that 10% of an adult's bones remodels in one year and bones are totally renewed every 10 years[23]. Bone is, therefore, a very dynamic tissue that is normally regenerated through the balance between the processes of bone resorption and formation that are regulated over time and space, as shown in Fig. 2.

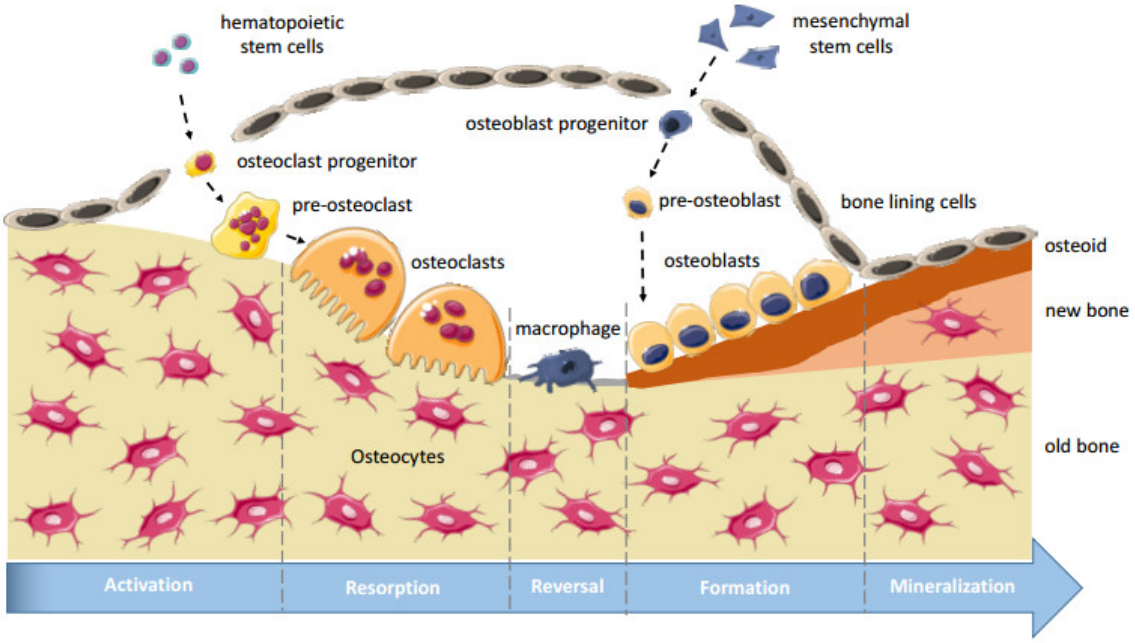


Fig.2 *- Bone turnover in which, through a balanced and regulated process, the osteoclasts reabsorb the bone and the osteoblasts deposit new bone*

Bone remodeling consists of a phase of resorption of the "old" bone, an intermediate phase and a phase of new bone formation. Each phase is checked in order to ensure the correct functionality of the bone[24]. In particular, bone resorption by osteoclasts precedes bone formation by osteoblasts[25]. Once an area of bone becomes a target for the degradation and removal of bone matrix, the osteoclasts migrate and bind firmly to the surrounding matrix establishing interactions between the bone matrix proteins and the polarized integrins, podomeres and actin filaments inside the osteoclasts and create a sealed area between the osteoclasts and the bone surface (resorption gap).

During the resorption process, several factors are released, including transforming growth factor β (TGF-β) or insulin-like growth factor (IGF), which are the main mediators of communication between osteoclasts and osteoblasts (Fig.2)[16,24]. In particular, TGF- β activates Runx2 through phosphorylation and activation of the SMAD protein (which can also be activated by BMPs)[26]. Runx2, which can also be activated directly by PTH and Fibroblast Growth Factor (FGF), is the most important transcriptional regulator in osteoblasts and is necessary for their differentiation. Its function is to initiate a precise and time-controlled gene transcription program: it forms multimeric complexes with DNA and its co-regulators in the nuclear matrix; subsequently the molecules with histone acetyl transferase activity remodel the chromatin to initiate the transcription of a defined gene expression profile[24].

Another important signal for osteoblast differentiation is Osterix (OSX). Like Runx2, its expression is regulated by the anabolic signals BMP-2 and IGF-1. OSX interacts with the Nuclear Factor of Activated T Cells (NFAT2), which must be dephosphorylated and activated by calcium-regulated calcineurin phosphatase / calmodulin to stimulate osteoblastogenesis and bone formation[27].

WNT-β-catenin signaling is another important pathway for both skeletal formation and skeletal remodeling. WNTs are a family of growth factors that bind to a membrane receptor complex consisting of one of ten FRIZZLED G protein-coupled receptors and one of two low-density lipoprotein (LDL) receptor-related proteins (LRPs). In particular, the best characterized is Lrp5, a single transmembrane domain receptor that interacts with the receptor complex to inhibit β-catenin phosphorylation by glycogen synthase kinase 3β (GSK-3β). If phosphorylated it is more susceptible to ubiquitin-mediated degradation; by inhibiting GSK-3β, LRP-5 causes the accumulation of β-catenin and its entry into the nucleus. Activated β-catenin cooperatively interacts with Tcf / Lef transcription factors to stimulate osteoblastogenic differentiation[28].

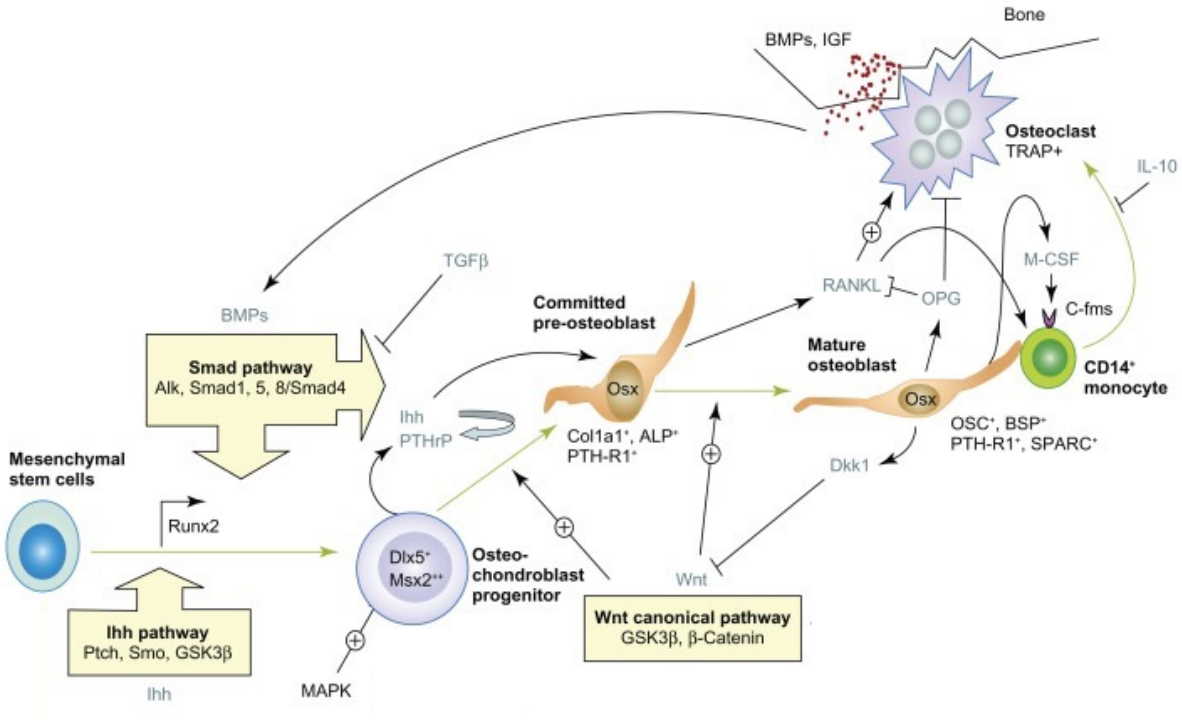


Fig 3 – *The signalling between osteoblast and osteoclast. Several Molecules secreted by these cells influence each other in a paracrine manner to maintain the balance of bone formation and bone resorption.*

Once the osteoblasts have differentiated, they stop proliferating, express ALP and begin secreting Col I and NCPs, remaining anchored to the newly formed protein matrix thanks to cadherin-11 and N-cadherin. Subsequently, the mineralization of the matrix takes place which

begins with the extrusion of nucleated vesicles, rich in Ca^{2+} and it is followed by the enzymatic cutting of the pyrophosphate, probably carried out by the ALP, which allows the formation of the first, even if imperfect, crystal nuclei. This subsequently grow by aggregation and addition of Ca^{2+} ions, in a process that is coordinated by phosphoprotein kinases and phosphatases that regulate the phosphorylation of the main nuclear phosphoproteins, BSP and DMP[16].

1.1.5. Pathologies characterized by bone loss

When resorption and the formation of new bone are no longer in equilibrium and there is an imbalance in favor of resorption, bone loss occurs (Fig. 4).

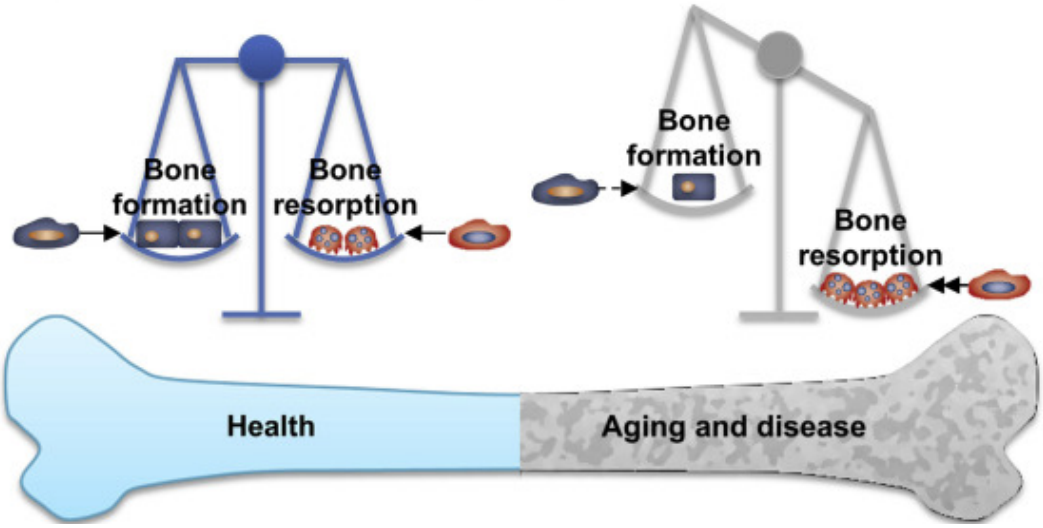


Fig. 4 *- Imbalance of bone remodeling in favor of bone resorption which causes bone loss*

Osteoporosis is defined as a systemic skeletal disease characterized by reduced bone mass and qualitative changes that are accompanied by an increased risk of fracture (as shown in Fig. 5).

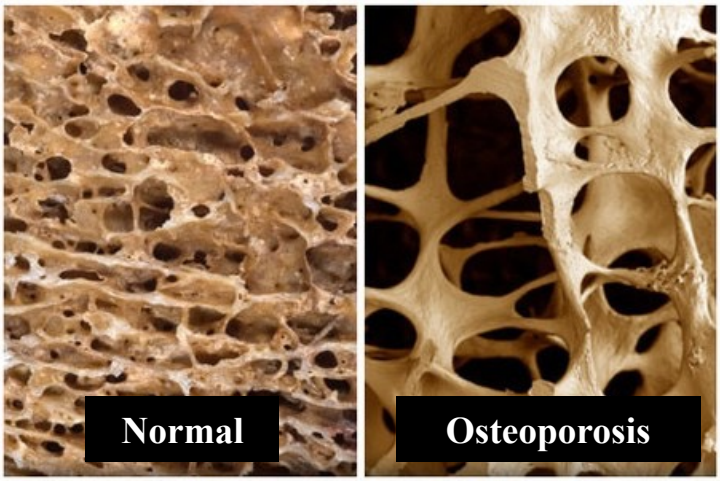


Fig.5 *- Comparison of images of normal bone and osteoporotic bone*

There are two forms of osteoporosis: Primitive and Secondary. The forms of osteoporosis that appear after menopause (postmenopausal osteoporosis) or with advancing age (senile osteoporosis) are defined as **Primitive**. While **Secondary** osteoporosis is caused by numerous pathologies and by the use of certain drugs[29].

Postmenopausal osteoporosis is the most common form of primary osteoporosis, resulting from the cessation of ovarian functions during menopause and from genetic and non-genetic factors that increase the impact of estrogen deficiency on skeletal tissue[30]. Initially this bone loss is limited by an increase in bone formation due to the expansion and proliferation of the pool of MSC cells and the increase in the differentiation of these pluripotent precursors towards the osteoblastic line[31]. The subsequent increase in osteoblast apoptosis[32,33], osteoclast number [34], oxidative stress[32] and inflammatory cytokine secretion (e.g. IL17 or TNF α), suppress the bone formation[35] and explain why bone formation does not increase as bone resorption.

The appearance of secondary osteoporosis can be due to numerous conditions, including smoking, alcohol, endocrine diseases (such as hypogonadism and hyperparathyroidism), haematological diseases (such as thalassemia and multiple myeloma), diseases of the gastrointestinal tract (such as chronic diseases liver disease and celiac disease) and rheumatic diseases (such as rheumatoid arthritis and psoriasis arthritis).

Other three important pathologies that can cause skeletal fragility and bone deformity are: Osteogenesis Imperfecta, Rickets and Osteomalacia.

Osteogenesis Imperfecta (OI) is a genetic disorder characterized by an increase in skeletal fragility, a decreased percentage of bone mass and a susceptibility to bone fractures of varying severity. Five forms have been identified. In 95% of cases, OI is due to mutations in the

COL1A1 and COL1A2 genes (17q21.33 and 7q21.3) which code for the alpha1 and alpha2 chains of type I collagen, the underlying protein structure of bone. All five clinical types of OI are due to to these mutations. Inheritance is autosomal dominant. Autosomal recessive forms of OI have been observed, due to mutations in the LEPRE1, CRTAP and PPIB genes (1p34.1, 3p22 and 15q21-q22). Autosomal recessive forms are always severe if associated with severe hypotonia.

Rickets and **Osteomalacia** are two equivalent pathologies that occurs in child and adult life respectively. They are caused by mutations in the gene producing the enzyme that converts 25-hydroxy vitamin D to the active form, 1,25-dihydroxy vitamin D, or in the gene responsible for the vitamin D receptor. This leads a delay in depositing calcium phosphate mineral in growing bones, thus leading to skeletal deformities in child, while in adult can lead severe fracture.

Some other skeletal disorders are not inherited but rather develop only later in life. One of the most common of these acquired skeletal disorders is a tumor of the bone. **Bone tumors** can originate in the bone (these are known as primary tumors) or, much more commonly, result from another tumor outside of the skeleton (these are known as metastatic tumors). Both types of tumors can destroy bone, although some metastatic tumors can actually increase bone formation. Primary bone tumors can be either benign (noncancerous), such as osteochondroma or malignant (cancerous), such as osteosarcoma and Ewing's sarcoma. Metastatic tumors are often the result of breast or prostate cancer that has spread to the bone. These may destroy bone (osteolytic lesion) or cause new bone formation (osteoblastic lesion). Breast cancer metastases are usually osteolytic, while most prostate cancer metastases are osteoblastic, though they still destroy bone structure. Many tumor cells produce parathyroid hormone related peptide, which increases bone resorption. This process of tumor-induced bone resorption leads to the release of growth factors stored in bone, which in turn increases tumor growth still further[36].

1.2.MUSCLE TO BONE CROSS-TALK

1.2.1. Skeletal Muscle as endocrine Organ

Skeletal muscle is a dynamic and plastic tissue of the human body, and it represents the 40% of body weight. Muscle tissue is mainly composed of water (75%) and proteins (20%) and other elements such as salts, minerals, and carbohydrates. Muscle mass is defined by the balance between protein synthesis and degradation and both processes can be influenced by external factors such as nutritional status, hormonal balance and physical activity [37].

Skeletal muscle has mechanical and biochemical properties. Mechanically, the muscle converts chemical energy into mechanical energy to generate strength and power to maintain posture and produce movement. From a biochemical point of view, muscle plays a key role in the body's energy homeostasis as it represents a reservoir of important substrates such as amino acids and carbohydrates, produces heat to maintain the body's core temperature and consumes most of the oxygen and fuel during training. physical activity and exercise[38].

Muscle tissue has a particular structure organized in bundles of muscle fibers covered by a connective tissue sheath. The skeletal muscle fibers are very large (they can have a diameter of 100 µm and length of the entire muscle, i.e., 30-40 cm) and multinucleated: during embryonic development, groups of cells called **myoblasts** fuse to create the single skeletal muscle fiber. Therefore, each nucleus of the fiber represents the contribution of a single myoblast. The cell membrane of a muscle fiber is called **sarcolemma**, while the cytoplasm is called **sarcoplasm**[37] (Fig.6).

Some myoblasts do not fuse with developing muscle fibers but remain in the adult muscle tissue in form of satellite cells, that are adult stem cells that contribute to growth, repair and regeneration of the tissue[39,40]. Satellite cells are normally quiescent but when stimulated by myogenic factors they proliferate and differentiate into new muscle fibers[41].

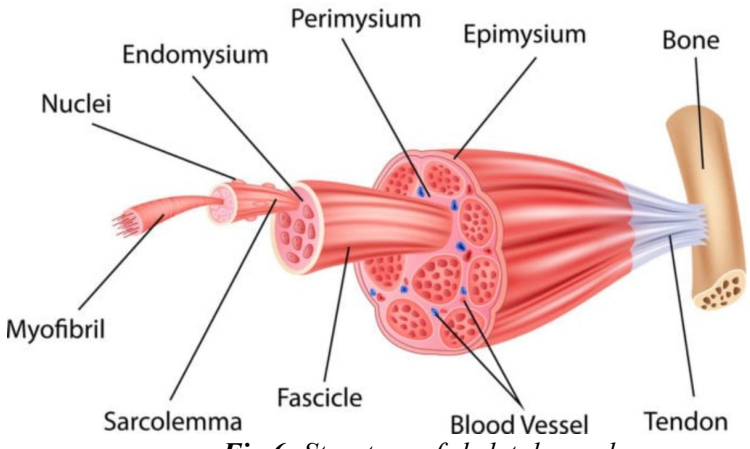


***Fig.6**- Structure of skeletal muscle*

Muscle contraction occurs following a stimulus, which is propagated through the nerve fibers and reaches the synaptic button where it releases the neurotransmitters. The neurotransmitter in turn triggers an action potential in the muscle fiber which in turn triggers a contraction.
The nervous system is not the only system through which the muscle communicates. After demonstrating that in subjects with paralyzed muscles there were the same physiological changes found in healthy humans [42], the need arose to study which molecules were released from the muscle and act in a paracrine and / or endocrine way, via the bloodstream. The first molecule to be identified was IL-6[43], Steensberg et al. demonstrated how the concentration of this cytokine increased in the bloodstream following physical exercise, carrying out its metabolic action in other areas of the body.
Following this discovery, the term "**Myokines**" was introduced to define all cytokines and other peptides that are produced, expressed and released by muscle fibers and exert either autocrine, paracrine or endocrine effects [44].
In addition to myokines, another category of molecules was subsequently discovered: The eserkines. Eserkines can be released within extracellular vesicles and can be nucleic acids, peptides, messenger ribonucleic acid (mRNA), microRNA, and mitochondrial deoxyribonucleic acid[45].
Myokines have a positive or negative effect on muscle function and general metabolism and include protein such as: myostatin (MSTN), small organic β-aminoisobutyric acid (BAIBA), meteorin-like (METRNL), various interleukins (IL- 6, IL-8, IL-7 and IL-15), irisin, Growth factor like fibroblast growth factor 2 (FGF2), insulin-like growth factor-1 (IGF-1) and leukemia factor (LIF). Myokines not only communicate with the same muscle but represent the mediators for communication with other organs: adipose tissue, bones, liver, pancreas, kidneys, and brain[46].

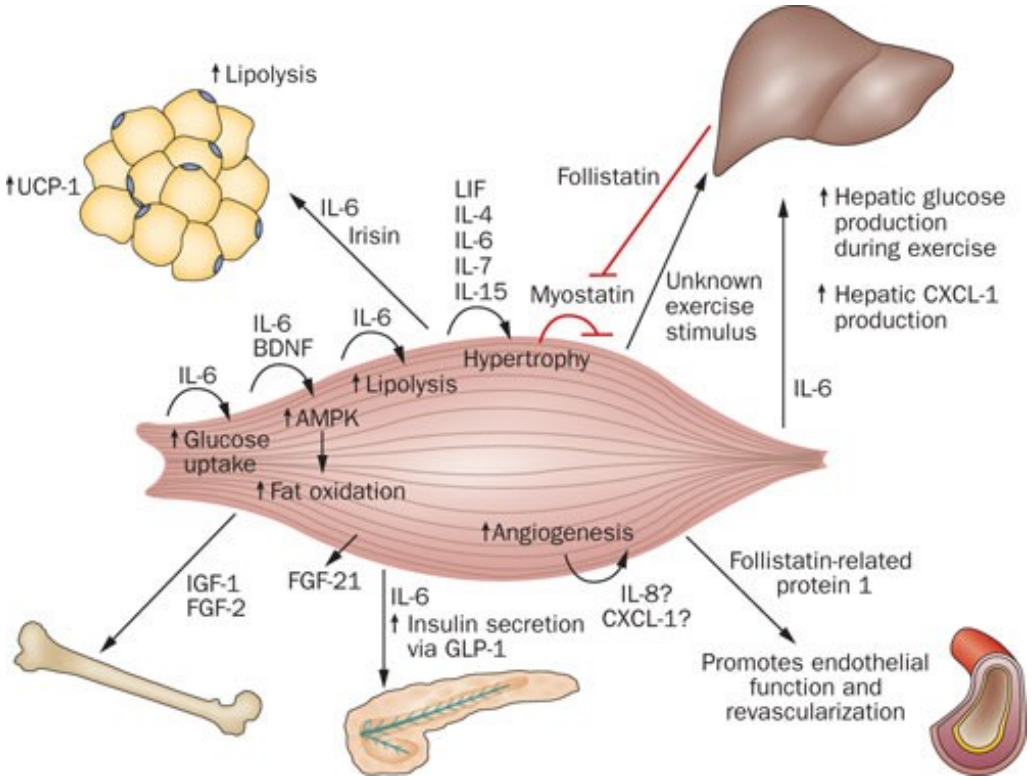


***Fig. 7** – Endocrine function of muscle*

- *Myostatin and other Growth Factor*

Myostatin, also called growth differentiation factor 8 (GDF8) belongs to the TGF-β superfamily. It is secreted into the bloodstream and its expression occurs mainly in skeletal muscle[47]. Myostatin is a dimeric protein synthesized in the form of precursors (Promiostatin) which must undergo a proteolytic cut in order to be mature [48]. Once mature, myostatin binds to peptides that keep it inhibited (such as Follistatin) and it is stored in the extracellular space, in latent complexes[49].

When myostatin is released, following various stimuli, it acts through its extracellular receptor (actRIIB), triggering a signaling pathway that leads the transcription regulation of target genes such as FoxO, Bix3 and Runx136[50]. In muscle, it acts as a negative regulator of skeletal muscle growth and development by inhibiting the activation and self-renewal of satellite cells and the proliferation of myoblasts.

Several evidence have shown that myostatin is a key regulator of body metabolism and is therefore correlated with the development of obesity, insulin resistance and type II diabetes[51–53].

Due to the established impact of myostatin signaling on muscle mass, coupled with the fact that myostatin is produced almost exclusively by skeletal muscle and that its presence is not essential for life, pharmacological interventions that target the myostatin signaling pathway

are relevant as potential therapies for muscle wasting diseases and metabolic muscle diseases[54].

In the category of growth factors insulin growth factor. 1 (IGF-1) and fibroblast growth factor-2 (FGF-2) can be included, whose activity is carried out mainly on bone and will be better described in the next paragraph.

- *Interleukins*

Numerous interleukins are produced by the muscle, including interleukins 6 (IL-6), IL-8, 7 (IL-7) and 15 (IL-15). IL-6 was the first protein to be defined as myokine, following the discovery that skeletal muscle can produce and secrete significant amounts of IL-6 into the bloodstream during exercise[55]. IL-6 is a pro-inflammatory cytokine, for this reason it was initially hypothesized that its synthesis was related to muscle damage due to exercise[56], but subsequent studies have shown that its synthesis occurs regardless of the presence of an inflammatory condition[57,58].

In fact, IL-6 acts as an energy sensor and the regulation of its expression is associated with the consumption of intracellular glycogen. During muscle activity, when the concentration of glycogen decreases because it is consumed by the cells to acquire energy, the expression of IL- 6 increase[59] . It is secreted into the bloodstream where it is responsible of important metabolic effects: it contributes to glucose production[60], improves the insulin-mediated glucose disposal process[61], modulates the fat metabolism [62](van Hall et al. 2003), increases lipolysis in skeletal muscle[59].

Another important interleukin recognized as myokine is IL-15, since it is accumulated in the muscle after exercise[63]. IL-15 seems to play different roles depending on the physiological or pathophysiological condition in which it acts. On the one hand it has an anabolic action, promoting the differentiation of myoblasts and increasing muscle mass by stimulating the production of contractile proteins[64], on the other hand systemic administration induces muscle atrophy[65]. Interestingly, in contrast to the postulated anabolic effects on skeletal muscle, IL-15 reduces the mass of adipose tissue. This reduction in fat mass could also be associated with the effects of IL-15 on liver metabolism: it decreases hepatic lipogenesis[66] (López-Soriano et al. 2004) and, at the same time, increases the oxidation of hepatic fatty acids[67].

Also, with regard to IL-8, its concentration increases during skeletal muscle contraction. The physiological function of IL-8 within the muscle is, however, still unknown. At the systemic level, the increase in IL-8 observed during exercise with an eccentric component is most likely due to an inflammatory response [68,69]. However, muscle-derived IL-8 is likely to manifest

locally and exert its effect in an autocrine or paracrine manner. A more likely function of muscle-derived IL-8 is to stimulate angiogenesis in skeletal muscle[70].

- *Irisin*

Irisin was recently classified as a myokine. It derives from the proteolytic cleavage of Fibronectin type III domain-containing protein 5 (FNDC5) and it is secreted into the bloodstream as an acting similarly as a hormone.

Irisin induces the browning of the white adipose tissue and stimulates thermogenesis. In particular, Irisin acts at the level of white adipose tissue cells by stimulating the expression of brown fat-like genes, including mitochondrial uncoupling protein-1 (UCP-1), causing the transformation towards a beige / brown phenotype and resulting in the gene expression and mitochondrial activity typical of brown adipose tissue[71–73] .

Interestingly, Irisin is expressed in muscle during physical activity and its expression is induced by PGC-1α[74](PPARγ coactivator-1 α) that is a transcriptional coactivator that mediates many biological programs related to energy metabolism and it is also expressed during exercise suggesting the existence of a positive self-regulation cycle between Irisin and PGC1α and suggesting that Irisin positively regulates muscle metabolism in an autocrine way [75].

Importantly, this causes a significant increase in total body energy expenditure and obesity-related resistance to insulin-resistance. Irisin could be a therapeutic protein for human metabolic disease and other pathologies that improve with exercise[76].

1.2.2. Bone-muscle unit

The skeletal muscle is closely connected to the bone, which provides it an attachment site and together work to make the movement happen: bone acts as a lever and muscle, through contraction, applies the force necessary to move the body [77]. The mechanical load imparted by the muscle has important consequences on bone metabolism and consequently also has systemic impacts. In fact, in these conditions, the bone signals the need for energy to support bone formation, while when it is not subject to load it signals the low energy requirement, in order to use the energy for other important activities [78].

Physical activity, which is an excellent example of mechanical communication between bone and muscle, affects skeletal muscle, bone tissue and strength; on the contrary, immobilization (e.i. paralysis) causes negative effects on both tissues. According to Frost's postulate, bone responds to mechanical stimuli by modifying its structure and its mass. In particular, a

mechanical stress threshold value is defined which, when exceeded, activates anabolic phenomena that lead to an increase in bone rigidity in order to prevent possible damage due to the increase in the applied force. When the applied force is lower than the threshold value, on the contrary, bone resorption is activated[79].
The mechanical strength of the muscle affects bone remodeling starting from fetal life, when the mechanical signals stimulate proliferation and differentiation towards the hypertrophic phenotype of chondrocytes[80–82]. The same phenomenon governs endochondral ossification during linear growth[82]. The shape of the bone is also determined by the mechanical load, its absence causes the reduction of the circumference and the integrity of the bone. Finally, in the absence of muscle activity, bone eminences are reduced or absent.

1.2.2.1. Biochemical interaction

The mechanical communication between bone and muscle is also associated with a biochemical type of communication through the production and secretion of various factors by muscle (myokines) and bone (osteokines) which act respectively on bone and muscle.

The muscle secretes several myokines that act at the bone level, regulating bone metabolism in a positive or negative sense. **Myostatin**, one of the main myokines, is a positive regulator of bone resorption, stimulates osteoclastogenic differentiation through RANKL by activating the nuclear factor of activated T cell (NFAT) signaling pathway [83]. At the same time, it acts reducing the formation of new bone both inhibiting the differentiation of mesenchymal stem cells into osteoblasts and stimulating the differentiation towards an adipogenic phenotype [84,85] and reducing the expression of osteogenic markers (such as ALP and Osterix) in osteoblasts[86].
In contrast, two important growth factors for bone tissue, IGF-1 and FGF2 mainly play an anabolic action by stimulating osteoblastic bone formation, and they are mainly produced by the muscle adjacent to the periosteum, in which their receptors are present[87]. In particular, IGF1 maintains the balance between osteoblasts and osteoclasts: it reduces apoptosis and stimulates osteoblastogenesis by activating the WNT/β-catenin signaling pathway [88]; stimulates osteoclastogenesis by improving the synthesis of RANKL[89].
Regarding **FGF2**, at skeletal level it induces bone formation, fracture healing and cartilage regeneration[87]. However, secreted FGF2 exists in two isoforms deriving from alternative splicing: high molecular weight and low molecular weight: the first acts in the nucleus as a transcriptional activator of genes that inhibit mineralization, such as SOST; the second is secreted in the extracellular space and acts by binding the FGFR 1 receptor to increase the

proliferation and differentiation of osteoblasts through the WNT/β-catenin signaling pathway [90]. Another important myokine that seems exert anabolic effects on bone is Irisin. Irisin promotes osteoblast differentiation through WNT/β-catenin signaling pathway and upregulates the expression of osteogenic gene such as OPN and SOST[91] . In the meantime, it also inhibits osteoclast formation[92,93].

Finally, proteins such as **IL-6** have functions on the bone that aren't yet defined, if on the one hand it seems to promote bone resorption[94], on the other it seems to regulate osteoblastic bone formation in a positive sense[95].

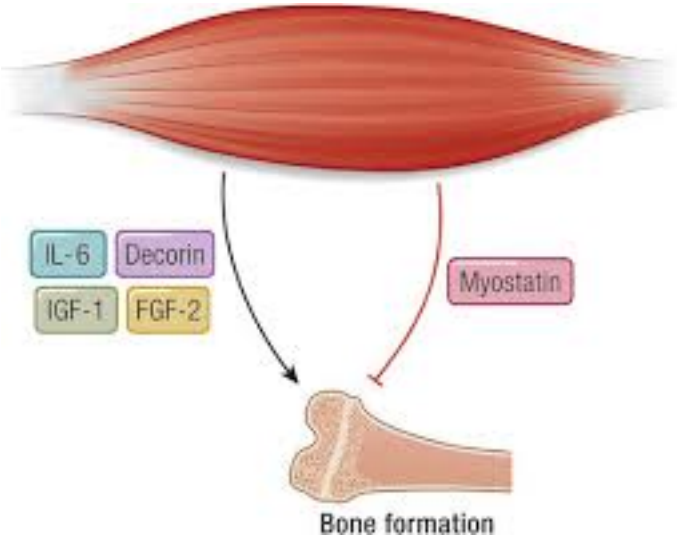


Fig 8 - *Muscle to bone cross-talk*

The bone is also able to communicate with muscle and other tissues by providing molecules known as osteokins including osteocalcin and sclerostin. **Osteocalcin** is an osteoblast-specific non collagenous protein, and it is involved in the regulation of male, fertility, insulin sensibility and energy metabolism. In muscle, it stimulates the release of IL-6 following aerobic excercise, it stimulates the proliferation of myoblasts and finally it is important for the maintenance of muscle mass and function [96–98].

SOST is a protein produced by osteocytes and its main function is to inhibit bone formation. At the muscular level, its function is not yet clear, its presence is however necessary for the correct functioning of the muscle. It was recently discovered that sclerostin is also produced by the muscle and works together with bone sclerostin, enhancing the negative regulation on osteogenesis[99].

1.2.3. Targeting bone-muscle crosstalk as potential therapeutic strategy

Many changes take place during aging that can affect health and life span. One of the biggest problems is the loss of muscle mass and the loss of bone tissue, phenomena known as

sarcopenia and osteopenia, which often occur together and are called sarcosteopenia. Sarcopenia is defined as the loss of strength, mass and function of the muscles associated with age. In this condition, the balance between protein formation (anabolism) and degradation (catabolism) is shifted towards catabolism.

Often associated with sarcopenia is osteopenia, a disease characterized by the destruction of the microarchitecture of the bone. These two diseases share many risk factors including age, endocrine disorders, diabetes and thyroid dysfunction. All of these conditions can alter the balance between bone and muscle by moving the muscles towards catabolism and the bones towards reabsorption and causing osteoporosis and sarcopenia. In addition, aging is characterized by a low inflammatory state that can affect the balance of muscle and bone through the secretion of pro-inflammatory cytokines and proteins, such as IL-6 which is mainly responsible for muscle atrophy.

The most accredited therapeutic strategy for the treatment of sarcopenia and osteosarcopenia is a lifestyle intervention based on nutrition and exercise, as it is a non-invasive and easily applicable strategy.

But since muscle and bone communicate and regulate their metabolic functions through the secretion of myokines and osteokines, targeting these molecules to stimulate one metabolic function (i.e. bone mass production) rather than another could represent an innovative therapeutic approach.

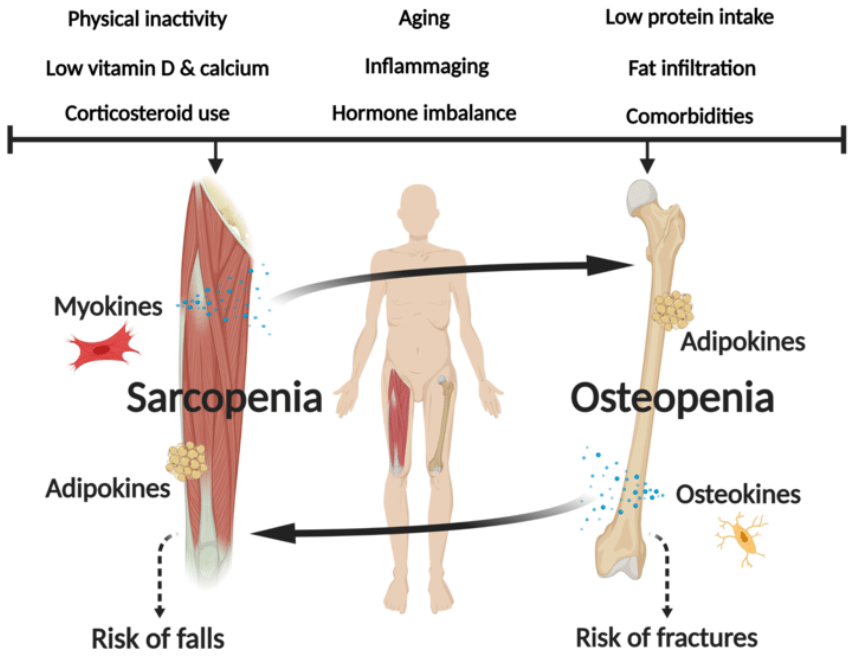


***Fig. 9**- Risk factors, muscle–bone crosstalk (myokines, osteokines, adipokines), and the pathophysiology of osteosarcopenia*

1.3. GALECTIN-3

1.3.1. Structural and biochemical characteristics of Galectin-3

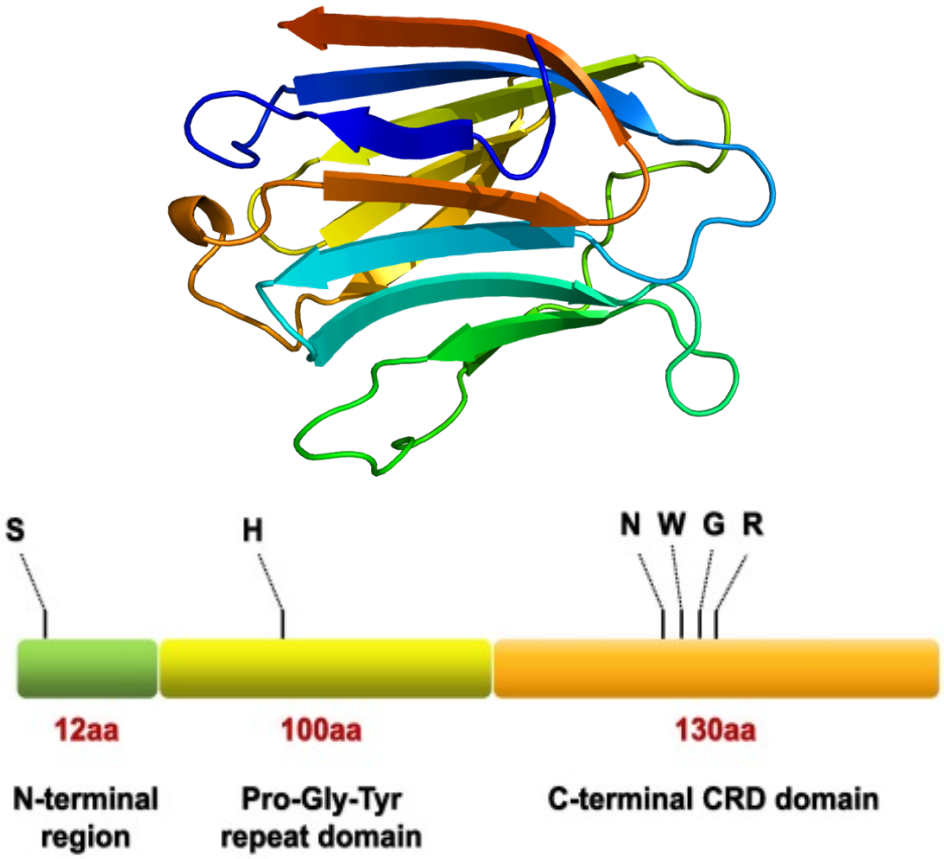


***Fig.10**-Structure of monomeric Gal-3*

Galectin-3 (Gal-3) is a 29- to 35 kDa protein and it belongs to the family of the β- galactoside binding lectins. Its polypeptide chain forms two distinct domains: an atypical N-terminal domain (ND) and a C-terminal carbohydrate recognition (CRD) domain (Fig. 10) and these two domains have not only structural, but also functional differences. The N- terminal domain is a flexible structure consisting of 110-113 amino acids and it contains multiple homologous repeats (7-14), that includes a consensus sequence Pro-Gly-Ala-Tyr-Pro-Gly, followed by three additional amino acids[100]. The ND domain has some important functions: it is essential for full biological activity of Gal-3 because it contributes together with the CRD in carbohydrate binding, and it is also involved in secretion of Gal-3 outside of cells through the initial 12 amino acid[101]. Finally, it is also involved in anti-apoptotic signaling activity.

The c-terminal CRD domain of Gal-3 consist of 130 amino acids, and it has a globular structure with five six-stranded β-sheet arranged in a β-sandwich[102]. It maintains the whole

carbohydrate-binding site within which it is contained the NWGR (Asp-Trp-Gly-Arg) motif that is a conserved amino acids sequence that is essential for Gal-3 binding to β-galactosides and it is involved in several biological activity of Gal-3.

Gal-3 is synthesized in cell cytoplasm, but it can shuttle between cytoplasm and nucleus: from one hand it can translocate in the nucleus thanks to its ND [103] while the other hand the movement from nucleus to cytoplasm involves a nuclear export sequence located within its CRD[104]. From cytoplasm, it can also be secreted outside the cells through a non-canonical secretory mechanism by membrane evagination. The secretion process initially involves the accumulation of gal-3 in the cytoplasmic side of the cell membrane through the heat shock proteins or other chaperonins and then the pinching off of revaginating mambrane and the release of the vesicle[105].

Gal-3 is synthetized and secreted in a monomeric form[106], but when it isn't bound to its ligand it is able to form homodimer by self-association through its CRD domain[107]. However, in presence of its carbohydrate binding ligands Gal-3 can polymerize to pentamers through its ND [107,108](Fig.11). This pentameric form, typical in extracellular space, allows Gal-3 to interact with the ligands of the cell surface to form lattice like structure and control some signaling pathway[109].

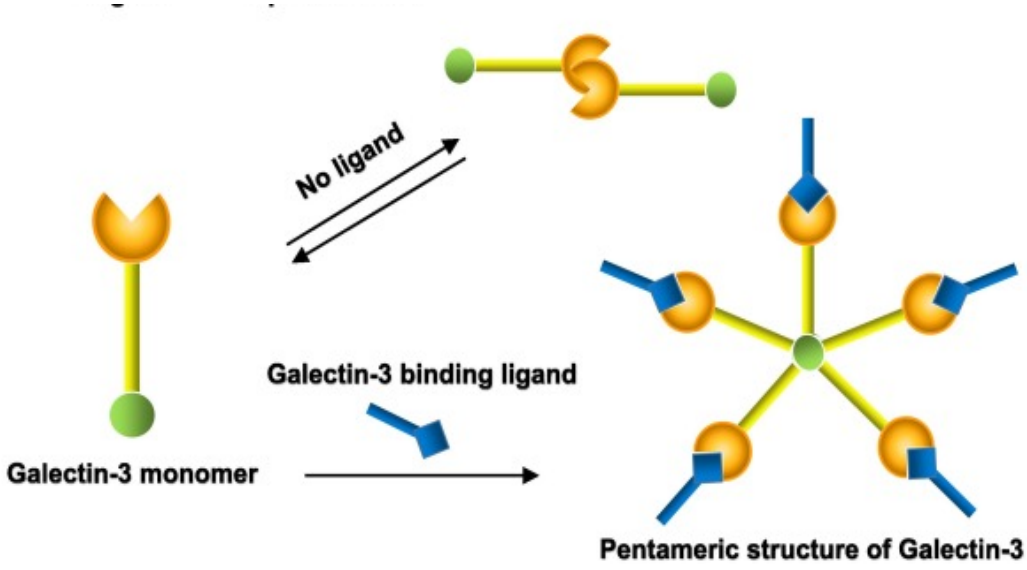


Fig.11 *– Schematic representation of monomeric structure of Gal-3, Gal-3 dimerization and Gal-3 polymerization*

The best-known Gal-3 ligands, for which it has a strong affinity, are Lactose (Lac), N-acetyl-lactosamine (LacNac) and glycoproteins which contain polylactosamines. Thanks to its affinity for polylactosamine glycans, some ligands of Gal-3 are: glycosylated extracellular matrix components (such as laminin, fibronectin and others), membrane proteins (such as

integrins) or lysosomal associated membrane proteins 1 and 2 (Lamp 1 e 2) (as reported in Tab.1). Anyway, Gal-3 is also able to interact with many intracellular proteins in a carbohydrate-independent manner[110].

	Ligand
Extracellular matrix proteins	Laminin
	Fibronectin
	Tenascin
	M2BP
Membrane proteins	Integrins:
	αM/β2 (CD11b/18)
	α1/β1
	N-CAM
	L1
	MAG
	LAMP-1,2
	MP20
	CD98
Intracellular Proteins	Cytokeratins
	Chrp
	CBP70
	Alix/AIP1
	Bcl-2
	Gemin-4
Others	AGE

***Table 1**- Ligands for Gal-3*

1.3.2. Functional properties of intracellular Galectin-3

Gal-3 is highly present in the cytoplasm and nucleus where it interacts with different ligands and affects different cell functions.

One of the first ligands to have been identified is **Bcl-2** with which it interacts through the CRD domain and regulates apoptosis. Bcl-2 is a protein located outside the mitochondrial membrane that regulates apoptosis by blocking the release of cytochrome-c from the mitochondrion. Together with Bcl-2, Gal-3 works as a mithocondrial-associated apoptotic regulator[111–113].

Cytosolic Gal-3 is involved in a positive regulation of cell proliferation, differentiation, survival and death thought its binding with **K-Ras** (an oncogenic protein)[114] and **AKT** protein[115,116] and this is also associated with a tumorigenic potential of Gal-3[117] (Fig. 12).

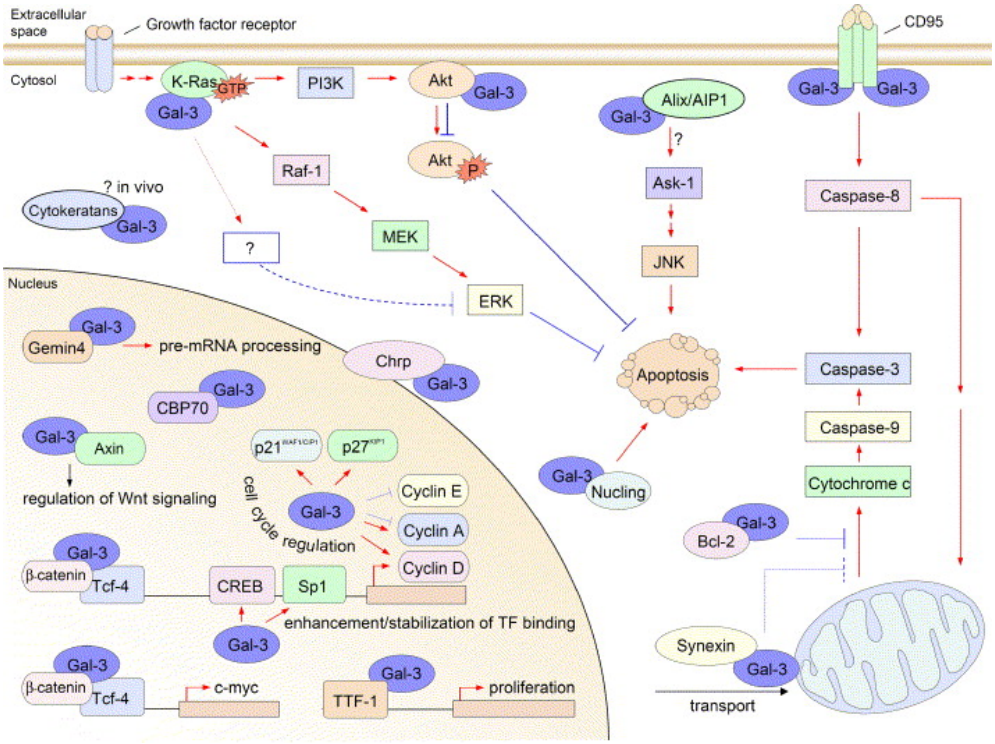


Fig. 12- *The intracellular functions of Gal-3.*
(Red arrows indicate positive effects, blue lines indicate negative effect)

In the nucleus Gal-3 has many binding partners and it act as regulator of different process. First, Gal-3 acts as pre-mRNA splicing factor indirectly binding the mRNA through **Gemin4** that is a part of gemini bodies (SMN complex) which function is assembly of spliceosomal snRNP and regeneration of spliceosomes required for pre-mRNA splicing in the nucleus[118].

Gal-3 regulates gene-transcription enhancing and stabilizing some transcription factor, such as CREB and sp1, in the promoter regions of many target gene including Cycling D1, cyclin A, cyclin E, p21 and p27[119,120], also suggesting a regulator role in cell cycle.

Moreover, Gal-3 is a binding partner of **β-catenin** and plays an important role in the regulation of wnt/β-catenin signaling pathway in two manners: from one hand Gal-3 replaces β-catenin, binds Axin and promotes GSK-3β phosphorylation[121], on the other hand Gal-3 binds the β-catenin/Tcf complex and induces the transcriptional activity of Tcf-4[122].

In the nucleus, the last important bond of Gal-3 occurs in a dependent carbohydrate manner and is with **CBP70**, a 70kDa glycoprotein[123,] that is a CREB-binding protein, and it is involved in transcription process.

1.3.3. Functional properties of extracellular Galectin-3

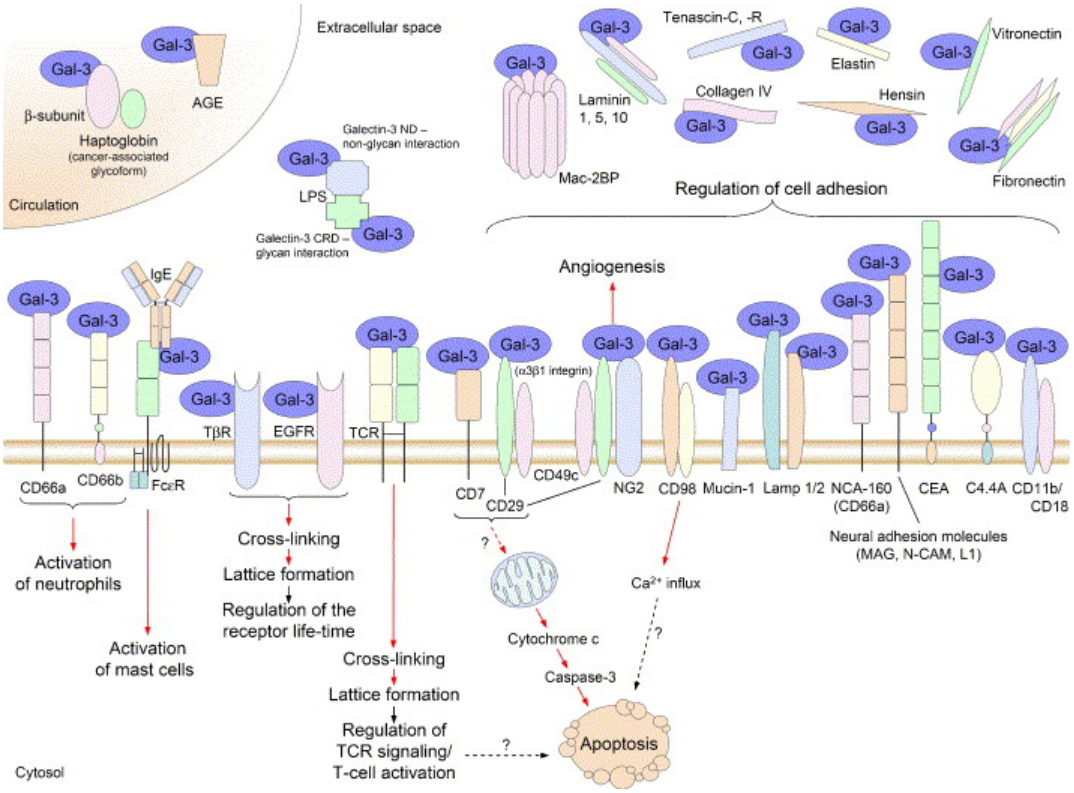


Fig.13- *The extracellular functions of galectin-3*

Gal-3 is present in the extracellular space where it can be localized on the cell surface, in the extracellular matrix or in biological fluids.

The extracellular Galectin-3 exhibits various autocrine and paracrine effects. The first effect is the modulation of cell adhesion thanks to the ability of Gal-3 to bind glycoproteins of the cell surface and glycosylated proteins of the cell matrix, including Laminin[124], Fibronectin[125], Hensin[126], Elastin[127], Collagen IV, Tenascin-C and R[128] and Integrins (i.e α1β1)[100]. This regulation can occur in positive or negative manner depending on the cell type, for example promote cell adhesion of human neutrophils to laminin and to endothelial cells, while in thymus it blocks thymocytes cell interaction[129]. Another important aspect is that, being involved in cell adhesion, Gal-3 is also a regulator of cell adhesion of cancer cells and it is involved in aggregation of tumor cells during metastasis[130].

The binding of Gal-3 to some cytoplasmic membrane proteins induces the activation of some signaling pathways that provoke different biochemical reaction in the cells, resulting in their activation. Monocytes and Neutrophils are two cell types in which Gal-3 is able to influence their activity. On the contrary, on myeloid cells Gal-3 can exert negative effect reducing IL-15 expression through FcγRII receptor. Furthermore Gal-3 induce angiogenesis in endothelial cells.

In addition to other roles, the extracellular Gal-3 acts as chemoattractant for monocytes and macrophages cooperating with G-protein-coupled cell surface receptor.

1.3.4. Galectin-3 state of art in bone tissue

Gal-3 is expressed in many tissues, including the bone, where is considered a marker of chondrogenic and osteogenic cell lineage. Different findings suggest that Gal-3 may be a key player in all stages of bone Biology.

Gal-3 shows an important role in mesenchymal stem cells, indicating that it is involved also in the early stage of osteoblastogenesis. In particular, Gal-3 enhance the osteogenic differentiation capability of MSC favoring β-catenin activity, competing for its GSK-3β site, which represent an important step in the degradation of this transcriptional coactivetor[131].

Another evidence is associated with bone remodeling. Study on LGALS 3 -/- mouse demonstrate an altered bone homeostasis due to a bigger activation of osteoclastogenesis suggesting that Gal-3 may be part a molecular mechanism through which osteoblast control osteoclastogenesis at site of mineralization[132].

An important process in which Gal-3 is involved is endochondral ossification. This is an essential process by which most bones are formed during embryonic development. Initially, endochondral ossification involves the formation of cartilaginous elements (chondrocytes) from mesenchymal condensation, the secretion of extracellular matrix from mature chondrocytes and the invasion of blood vessels. This growth tissue is localized at the metaphyseal level and the chondrocytes are organized in three zones: proliferative, mature and hypertrophic. A series of highly coordinated events then occur which include apoptosis of chondrocytes in the hypertrophic zone, degradation of the calcified cartilage matrix and deposition of the matrix by osteoblasts on the remaining cartilage septa, which act as scaffolds[133].

In this scenario, the Gal-3 has several roles; first of all, high concentrations of Gal-3 were found in mature chondrocytes and low concentrations in hypertrophic chondrocytes, confirming the anti-apoptotic action of Gal-3 and the maintenance of cell survival. [134]. Studies in LGAL-/- mice confirmed that Gal-3 participates as a regulator of chondrocyte survival and also participates in the coordination between chondrocyte death and metaphyseal vascularization, while have not impact on bone elongation[135].

Furthermore, Gal-3 levels are also elevated in osteoblasts, osteocytes and osteoclasts, suggesting a role of this lectin in the differentiation and activity of these cells in mature tissue[134].

Early evidence of the role of Gal-3 on bone tissue showed that osteoblasts and chondrocytes share Gal-3 expression in addition to other osteogenic markers such as ALP or OCN or BSP and during osteogenic differentiation, Gal-3 is highly expressed in mature osteoblasts that form bone nodules[136].

Subsequently, the same author has reported the most striking evidence in favor of a key role of Galectin-3 in osteogenic differentiation. It was observed that mRNA levels and protein levels increase during differentiation and it also has been confirmed that this increase occurs concurrently with the increase in osteogenic markers[137]. Another important finding is that Gal-3 is RUNX2 target gene in bone[138]. But the mechanism through Gal-3 may regulate differentiation and activity of osteoblast remain to be determinated.

All these data demonstrate that Gal-3 has a crucial role for osteoblast differentiation and function, but a recent report demonstrate that exogenous Gal-3 has opposite effect, it inhibits the terminal differentiation of the pre-osteoblast cell-line[139] . These findings could suggest that Gal-3 has opposite effects on osteoblastogenesis depending on its intracellular or extracellular location, as described above

2. Aim of the work

Galectin-3 is a protein widely expressed in different types of cells and tissues and acts both intracellularly and extracellularly interacting with different target proteins and affecting numerous biological processes and different physiological and pathophysiological conditions. It has been shown to be a key protein in all stage of bone biology: it is classified as a marker of osteogenesis as its expression increases during osteogenesis, and it is widely present in both developing and mature bone and its expression is controlled by the main regulator of bone growth, RUNX2.

Bone tissue is a highly dynamic tissue characterized by continuous processes of resorption of the "old" bone and formation of "new". These two processes are constantly in balance and finely controlled in an autocrine and paracrine way. In close contact with the bone there is the muscle, which communicates with the bone not only mechanically, applying the force necessary for movement through contraction, but also biochemically, through the production of endocrine factors called myokines, that participate to the formation, repair and maintenance of bone.

In turn, the bone produces chemokines, which act on the muscle, called osteokines. This molecular and biochemical interaction is called "bone-muscle cross-talk".

In order to identify new myokines that may be new therapeutic targets for the benefit of the muscular system but especially for the skeletal system, particularly in old age, the initial aim of our study was to define new myokines that modulate osteogenesis. Using a proteomic approach based on mass spectrometry, several myokines were revealed in a medium obtained from human and mouse myotubes cultured in different stages of myogenic differentiation. By comparing the different proteins obtained, we identified some interesting proteins that appear to have a regulatory function during bone processes, including Gal-3.

For this reason, our study proceeded with the goal of defining the role of Gal-3 on bone tissue and its possible involvement in skeletal muscle cross-talk.

First, we characterized Gal-3 expression in vitro, during myogenic differentiation and osteogenic differentiation, and in vivo, in several growing mouse muscles.

We then investigated the effect of recombinant Gal-3, which mimics extracellular Gal-3 secreted by muscle cells, during osteoblast differentiation. In order to identify the main classes of proteins modulated by Gal-3, we analyzed the entire proteome of differentiated osteoblasts in the presence of recombinant Gal-3, by mass spectrometry. We then evaluated the signaling mechanisms downstream of the extracellular stimulation of Gal-3, both with a protein array approach based on phosphorylated proteins and metabolic molecules involved in bone metabolism. Furthermore, through an ECM

Adhesion Array, we monitored the impact of Gal-3 on the ability of osteoblasts to interact with extracellular matrix proteins, evaluating specific cell surface integrins and adhesion molecules.
Finally, to demonstrate that these findings are related to extracellular Gal -3 produced by muscle cells, we performed co-culture experiments between myoblasts and osteoblasts in which muscle cell Gal-3 was overexpressed.
In this thesis, for the first time, the molecular mechanism through which extracellular Gal-3 acts on bone tissue is extensively described, with particular reference to Gal-3 produced by muscle. The definition of the mechanisms of action of Gal-3 will allow to identify new therapeutic and pharmacological approaches for the treatment of musculoskeletal diseases, especially in those pathological conditions (i.e. aging and inflammation) where there is a greater secretion of Gal-3 which negatively affects bone metabolism.

3. Materials and Methods

3.1. EXPERIMENTAL WORKFLOW

In Fig.14 is schematized the experimental workflow. In particular, we show the detailed experimental time course and the moments when were carried out the medium changes, the treatments and the assays regarding the experiments of:

- *Broad analysis of secreted proteins of C2C12*

In this experiment, the C2C12 myogenic cells were seeded on a plate in normal culture medium and the following day (day 0) the medium was changed with differentiating medium. Subsequent medium changes were performed on: day 1, day 4, day 6, day 10 and day 14, as shown in **Fig.14 A**. The medium recovered at each time point was stored and analyzed by mass spectrometry to identify all possible myokines produced by myogenic cells during differentiation.

- *Characterization of three new putative myokine during osteogenic and myogenic differentiation*

Three osteogenic cell lines (Mesenchymal Stem Cell (MSC), Human osteoblast (HOB), Mouse pre-osteoblast (3T3)) and three myogenic cell lines (mouse satellite cells (C57), Human myoblast (LH), Mouse myoblast (C2C12)) were used in this experiment. The seeding of these cells took place on a plate in normal growth medium and the following day (day 0) the medium was changed with differentiating medium to induce respectively: osteogenic differentiation and myogenic differentiation. Subsequent medium changes were performed every 3-4 days and are shown in **Fig.14 B**. To verify the differentiation, at different experimental times, photos were taken to show the morphological change of myogenic cells into myotubes, while staining with Alizarin Red was performed for osteogenic cells. Protein extraction was carried out at the same time points to analyze the expression of Osteoglycin, Decorin and Galectin-3 by Western Blot (**Fig. 14C**).

- *Characterization of Galectin-3 in vivo in mice at different ages*

In this experiment, we analyzed the expression of Galectin-3 in three different muscles (Trapezius, tibialis and Gastrocnemius) of three mouse at different ages: young, adult, old (**Fig. 14D**).

- *Effect of extracellular Galectin-3 during osteogenic differentiation*

In these experiments we proceeded to seeding the cells on a plate with normal culture medium and the following day (Day 0) the medium was changed with an osteoinductive stimulus and the treatment or without it:

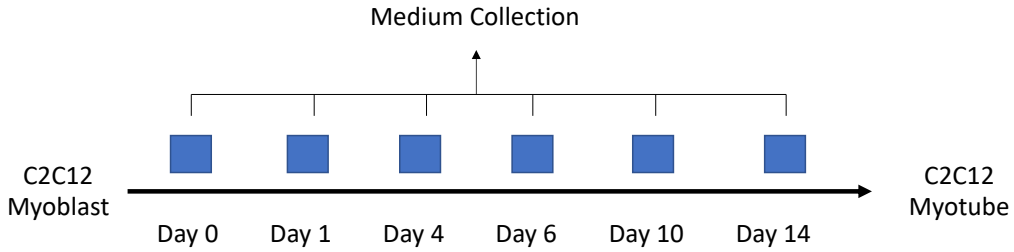
- 3ug / mL of recombinant Gal-3, which mimics Gal-3 produced by muscle cells.

Medium changes were performed every 2-3 days.

To verify the progress of osteogenic differentiation, at the different experimental times, shown with the **green square in Fig.14E**, the staining with alizarin red and the alkaline phosphatase assay were performed.

In addition, the following assays were performed to understand the molecular events downstream of stimulation with Galectin-3 or 4uM of TD139, a Galectin-3 inhibitor: Mass spectrometry, to study the entire proteome of cells, the adhesion assay to evaluate the ability of these cells to interact with the extracellular matrix, the bone metabolism array to evaluate the protein expression involved in bone metabolism and the Reverse Phase Protein Array (RPPA) to identify the signaling pathways activated or deactivated by the treatment.

A



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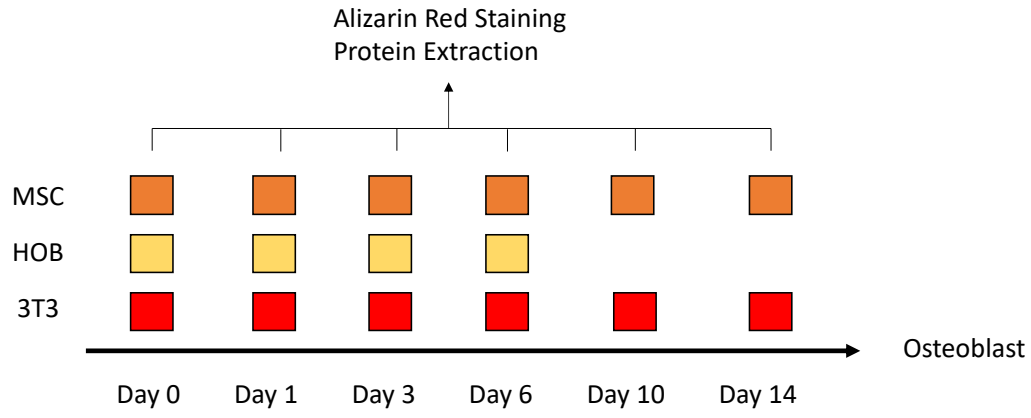

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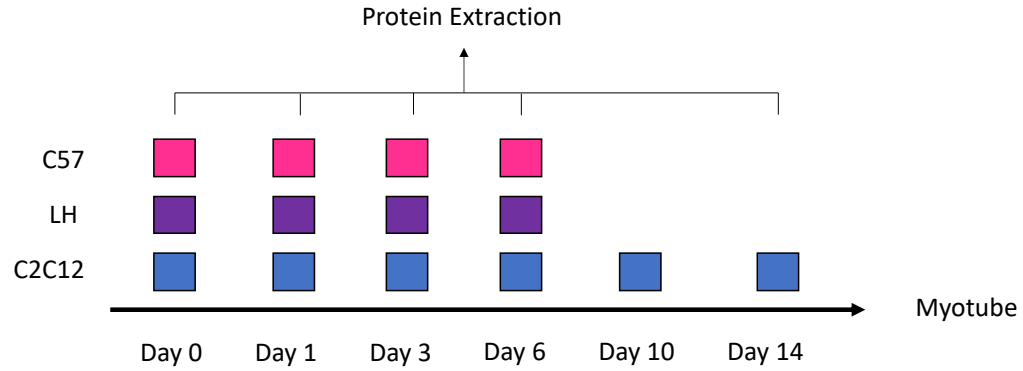

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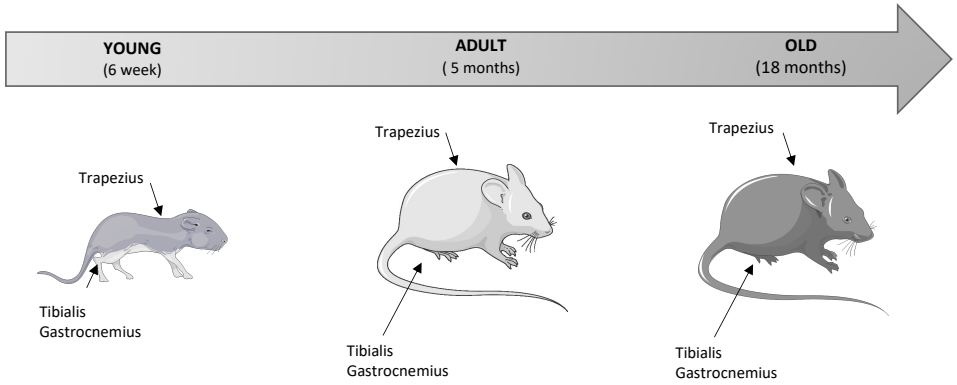

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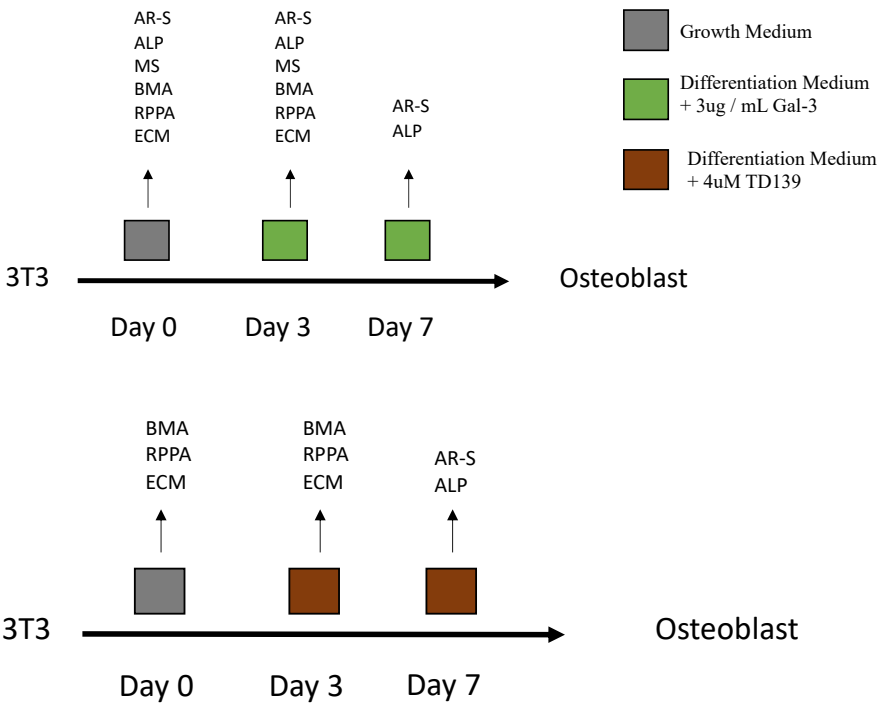


***Fig. 14-** Experimental workflow*

3.2. CELL CULTURE

Three osteogenic cell lines were used: Mesenchymal Stem Cell (MSC), Human osteoblast (HOB), Mouse osteoblast (3T3); Three myogenic cell lines were used: mouse satellite cells (C57), Human Myoblast (LH), Mouse Myoblast (C2C12). The cells were cultured under sterile conditions in flasks, in different growth medium depending on the cell type, as shown in **Table 2**.

Cell Type	Growth Medium
C57	20% Fetal Bovine Serum (FBS) 1% Pen-strept 1% L-glutamine (L-Glu) 88% HAM'S F12 +2,5 μg/mL Fibroblast Growth Factor (FGF)
C2C12	10% FBS 1% Pen-strept 1% L-Glu 88% Dulbecco's Modified Eagle Medium (DMEM)
LH	20% (FBS) 1% Pen-strept 1% L-Glu 88% HAM'S F12 +2,5 μg/mL FGF
Mesenchymal Stem Cells (MSC)	2,5% PLP 0,2% Heparin 1% Pen-strept 1% L-Glu 75,3% Minimum essential Medium α-modification (α-MEM)
Human Osteosblast (HOB)	10% FBS 1% Pen-strept 1% L-glu 88% α-MEM
Mouse Osteoblast (3T3)	10% FBS 1% Pen-strept 1% L-Glu 88% α-MEM

Table 2

The cells were kept in an incubator at a temperature of 37° C and in a humidified atmosphere composed of CO_2 (5%) and air (95%). Each operation / treatment carried out on the cells was performed in sterile conditions thanks to the use of a class II laminar flow hood and equipped with HEPA filters. Cell growth was monitored by observing the confluence under a microscope (Nikon) and carrying out cell counts using the Burker chamber.

Once, approximately 80% of confluence was reached, the cells were washed with 1X PBS and detached with 1X trypsin for 5 minutes (0.25% Trypsin, 0.1% EDTA, Corning). After blocking the action of trypsin, the cell suspension was collected in a falcon and centrifuged at 1200 rpm

for 5 min at RT. The pellet was resuspended in an adequate volume of growth medium and were counted. Based on the number obtained, the cells were re-seeded to be differentiated.
The cells were counted and seeded in 6 or 12 well plates according to the experiments in growth medium at density of 10.000 cells /cm^2 and subsequently cultured for the predefined experimental times in differentiation medium, described in **Table 3**:

Cell Type	Differentiation Medium
C57	2% Horse serum 1% Pen-Strept 1% L-Glu 94% DMEM
C2C12	2% Horse serum 1% Pen-Strept 1% L-Glu 94% DMEM
LH	2% Horse serum 1% Pen-Strept 1% L-Glu 94% DMEM
Mesenchymal Stem Cells (MSC)	10% FBS 1% Pen-strept 1% L-Glu 10 mM Dexanetasone 0,1 mM Ascorbic Acid 10 mM β-glycerophosphate 88% α-MEM
Human Osteosblast (HOB)	10% FBS 1% Pen-strept 1% L-Glu 0,3 mM Ascorbic Acid 4 mM β-glycerophosphate 88% α-MEM
Mouse Osteoblast (3T3)	10% FBS 1% Pen-strept 1% L-Glu 0,3 mM Ascorbic Acid 4 mM β-glycerophosphate 88% α-MEM

Table 3

At each experimental time the pellets and the culture mediums were collected for the subsequent experiments.

3.3. CHARACTERIZATION OF GALECTIN-3 EXPRESSION IN MICE OF DIFFERENT AGES

Eighteen male C57BL/6 mice at the age of 6 weeks (young; n=6), 5 months (adult; n=6) and 18 months (old; n=6) were purchased from Janvier Labs (Saint Berthevin, France). All animal procedures were performed in accordance with the European Guidelines for Care and Use of

Laboratory Animals. Animal housing and caretaking were provided by the animal facility in accordance with the national guidelines.

All animals were kept under a 12 h–12 h light/dark cycle and the temperature was controlled. After the first week of adaptation, animals were euthanized by cervical dislocation and the following muscles were isolated: Gastrocnemius, Trapezius and Tibialis; Femur was taken as positive controls.

3.4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Galectin-3 levels in animal tissues were determined with a quantitative sandwich ELISA. Galectin-3 ELISA kit (Galectin-3 Mouse SimpleStep ELISA Kit, Abcam) was used according to the standard protocols provided by the manufacturer. All samples were assayed in triplicate.

3.5. ALIZARIN RED-STAINING (AR-S)

Alizarin red (AR) is a dye based on alizarin, an organic compound synthesized from anthracene and capable of binding various elements, including calcium, generating a red / purple color. Alizarin red stain was used to label calcium salts deposited by pre-osteoblasts to monitor osteogenic differentiation.

At the end of each experimental time, the monolayer cells were fixed with 4% paraformaldehyde for 20' under constant agitation. After removing the fixative and performing two washes with 1X PBS, the AR dye was added to each well for 5’ at RT under stirring. At the end of the incubation, the dye was eliminated, three washes were performed in 1X PBS under constant agitation for 5’, each in order to remove the residues of non-specific staining and finally the plates were dried in the air. The level of mineralization was observed under the microscope and representative images were captured.

The deposition of calcium salts was quantified through the solubilization of alizarin red in *cetylpyridinium chloride* (CPC), adding it to the colored wells for 30' under stirring, after which the absorbance of the colored CPC was measured with a spectrophotometer at a length of 'wave of 570 nm.

3.6. ALKALINE PHOSPHATASE (ALP) ASSAY

Alkaline phosphatase (ALP) is a hydrolase enzyme that catalyzes the removal of phosphate groups in a basic environment. It is produced by osteoblasts, and it is involved in the formation and healing of bones. The alkaline phosphatase test is a colorimetric assay that allow to measure

the activity of the enzyme present in each biological sample, using a substrate, paranitrophenyl phosphate (PNPP), which turns to yellow color when it is dephosphorylated by the enzyme.
The test involves the preparation of a standard curve (in which the samples contain increasing amounts of 1mM pNPP and a fixed amount of ALP) and the preparation of the samples to be analyzed. For the preparation of the standard curve, we proceeded by preparing the different dilutions of the pNPP according to Table 4 and aliquoting 120 µL of each sample in a 96-well plate.

Standard	pNPP 1mM Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount pNPP in well (nmol/well)
1	0	300	120	0
2	10	290	120	4
3	20	280	120	8
4	30	270	120	12
5	40	260	120	16
6	50	250	120	20

Table 4

For the preparation of the sample, we proceeded by lysing the cell pellets by adding a volume of *ALP assay buffer* suitable for the size of the pellet, centrifuging for 15 'at 12000 rpm in order to eliminate the insoluble particles and transferring the supernatant in which the enzyme is present in new eppendorfs. At this point, several dilutions of the sample have been prepared (to ensure that the sample reading falls within the calibration curve) directly in the well of the 96-well plate bringing to a final volume of 80 µL with the *ALP assay buffer*. Each sample was prepared in triplicate. Once the plate was loaded, 50 µL of 5 mM pNPP were added to the wells of the sample to be analyzed and 10 µL of ALP enzyme in the samples of the standard curve. The plate was incubated for 60' in the dark at RT, a time in which the enzyme converts the pNPP substrate in equal quantities into colored paranitrophenol (PNP). At the end of the incubation, 20 µL of *stop solution* were added to block the reaction and the plate was read on the spectrophotometer at a wavelength of 405 nm.
Once the absorbances were obtained, the standard curve was constructed: the average value of the white absorption is subtracted and the average of the two readings of each standard is made. A graph is constructed in which the pNPP concentration is related to the absorbance and the "best fit" line is drawn to build the calibration curve, then the equation of the curve is calculated.
The ALP activity in the sample was obtained by applying the following equation:

$$\frac{B}{\Delta t * V} * D$$

Where:

- B is the amount of pNPP in the sample which is obtained by averaging the leyyuras of each sample, subtracting the average value of the blank absorption and interpolating this value in the calibration curve.
- T is the reaction time
- V is the volume of sample added to the well
- D is the sample dilution factor

3.7. EXTRACELLULAR MATRIX ADHESION ASSAY

The objective of the experiment was to evaluate the adhesion capacity of cells towards 7 specific ligands of the extracellular matrix immobilized on a support:

- Collagen I (Col I)
- Collagen II (Col II)
- Collagen IV (Col IV)
- Fibronectin (FN)
- Laminin (LM)
- Tenascina (TN)
- Vitronectin (VN)

The cells will be able to interact with these ligands only by expressing specific receptors on their surface.

For this assay, ECM Cell Adhesion Array Kit (ECM540 Sigma-Aldrich) was used. It consists of a 96 well plate with 12 strips. Each strip contains 8 wells each coated with a different extracellular matrix protein and one with BSA (CTRL -).

Col I	Col I	Col I	Col I	Col I	Col I	Col I	Col I	Col I	Col I	Col I	Col I
Col II	Col II	Col II	Col II	Col II	Col II	Col II	Col II	Col II	Col II	Col II	Col II
Col IV	Col IV	Col IV	Col IV	Col IV	Col IV	Col IV	Col IV	Col IV	Col IV	Col IV	Col IV
FN	FN	FN	FN	FN	FN	FN	FN	FN	FN	FN	FN
LM	LM	LM	LM	LM	LM	LM	LM	LM	LM	LM	LM
TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
VN	VN	VN	VN	VN	VN	VN	VN	VN	VN	VN	VN
-	-	-	-	-	-	-	-	-	-	-	-

Fig.15- *Schematic representation of 96 well plate of cell adhesion array*

We proceed with the rehydration of the strips with 200 μL of PBS 1X for 10' at RT. At the same time, the cell suspension was prepared: the cells were detached in a non-enzymatic way, using 5

mM of EDTA for 10 'at RT. Once the cells have been detached, after having eliminated the EDTA through a centrifuge and carried out a washing in 1X PBS, they were counted with the Burker chamber and a volume of assay buffer was added such as to bring the cell concentration to 2 * 10^6 cells / mL. Aliquot 100 µL of cell suspension was put into each well and incubated at 37 ° C for 2h, time in which the interaction of the cells with the ligands will occur or not. After incubation, the cell suspension was removed from each well, 3 washes were carried out with 200 µL of *Assay buffer* and finally 100 µL of *Cell staining solution* were added for 5 'at RT. After staining, three washes were carried out to eliminate the non-specific dye. Since the cell staining solution was Crystal violet dye which binds DNA it is possible to quantify the cells that have interacted with the ligands by adding the Extraction Buffer which solubilizes the dye and read the absorbance at 570 nm on the spectrophotometer.

3.8. PROTEIN EXTRACTION AND QUANTIFICATION

At each time point, described in par. 3.1, The cells were collected and stored at -20 ° C in the form of cell pellets. On the day of the protein extraction, the pellet was solubilized in an adequate volume of Lysis Buffer consisting of 20 mM of Tris-Cl and 150 mM of NaCl, which together form a buffer system, 10% glycerol, 1 % NP-40 (membrane breaker), 20 mM NaF (phosphatase inhibitor), 10 mM EDTA (Ca^{2+} chelator). Protease inhibitor (Benzamidine 0.5 mg / mL, Aprotinin 2 µL / mL, Leupeptin 2 µL / mL and PMSF, Phenylmethanesulfonyl Flouride, 0.75 mM) was added to this Buffer. Subsequently it is incubated for 20 minutes at 4 ° C. Then, a centrifuge is carried out at 12000 g for 15' at 4 ° C. Finally, the supernatant, consisting of the protein extract, is removed, and transferred to a new eppendorf. The extracted proteins can be stored at –80 ° C until use.

The extracted proteins were quantified with the Bradford method, which is based on the use of the *Comassie Blue G-250 dye*, which binds specifically to residues of arginine, tryptophan, tyrosine, histidine and phenylalanine, giving a color that varies from red to blue. The dye binds to these residues in anionic form, giving rise to an adduct with maximum absorbance at 595 nm. The concentration was calculated with respect to a calibration curve carried out with a standard protein of known concentration, bovine albumin (BSA).

3.9. PROTEIN EXPRESSION ANALYSIS BY WESTER BLOT

Thanks to this technique, it is possible to obtain qualitative or semi-quantitative data relating to the distribution and expression levels of a specific protein within a protein extract. The

identification of the protein is performed using an antibody that reacts specifically with the protein itself, previously immobilized on a solid support, such as a nitrocellulose membrane.
The levels of the following targets were analyzed in this study:

- OSTEOGLYCIN
- DECORIN
- GALECTIN-3
- ALP

3.9.1. Protein Electrophoretic Migration

The extracted and quantified proteins were separated by electrophoresis on a polyacrylamide gel, in conditions such as to ensure the loss of their tertiary structure so that the migration is exclusively a function of their molecular weight. The separation takes place in an electric field and in presence of Sodium Dodecyl Sulfate (SDS), an anionic detergent that denatures the tertiary protein structure by binding to the polypeptide in proportion to its molecular weight. Thanks to the use of a marker consisting of a mixture of proteins of known molecular weight, it is possible to estimate the molecular weight of the polypeptide chain of interest. Two different gels were prepared for pH and ionic strength: the stacking gel, placed at the top, which allows to concentrate the proteins deposited in the wells along a linear front for migration, composed of a percentage of acrylamide-N, N'-methylenebisacrylamide of 4%; the separating gel, located at the bottom, which allows the actual separation of proteins through a percentage of Acrylamide-N, N-methylenebisacrylamide which is a function of the molecular weight of the protein to be analyzed.
Before loading, the sample must be denatured by boiling it for 5 'at 100 ° C. The run, conducted at a current intensity of 120 V, look place in a Power-Pac 3000 (BioRad) instrument in the presence of a 1X Running buffer added with 20% SDS.

3.9.2. Transfer of proteins from the gel to the nitrocellulose membrane

After the electrophoretic run, the proteins contained in the gel were transferred to a solid support in order to be visualized with the specific antibody. The polyacrylamide gel was placed in contact with a sheet of 3 mm paper of the same size as the gel and soaked in Transfer Buffer, the solution in which the gel and the nitrocellulose membrane will be immersed during the transfer. The nitrocellulose membrane (Nitrocellulose Membrane 0,45 μm, BioRad) then rests on top of the gel, previously equilibrated in bidistilled H_2O and then immersed in the Transfer Buffer for 10'. Over the membrane spread another sheet of 3 mm paper. Subsequently, the sandwich (Fig. 3), formed by 3 mm paper-gel-nitrocellulose-3mm paper,

was placed in the semi-dry transfer (Trans-Blot® SD Semi-Dry Transfer Cell, BioRad) in such a way that the gel containing the negatively charged proteins, both on the cathode side, while the nitrocellulose membrane, towards which the proteins must be transferred, faces the anode. The transfer took place at 10V for 30 '.

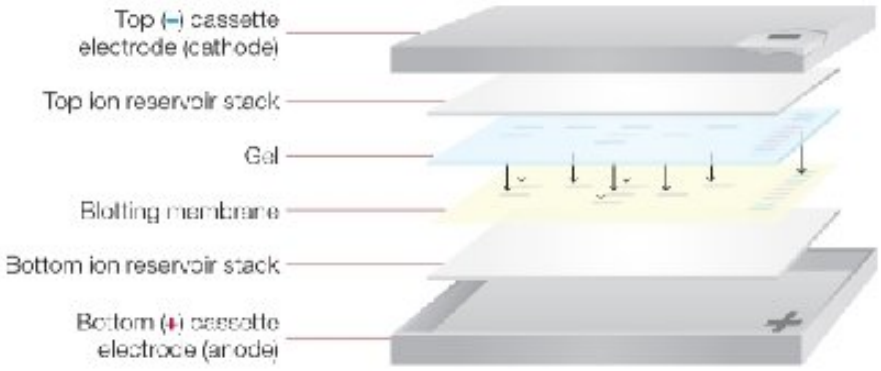


Fig. 16 - *Representation of gel-nitrocellulose membrane transfer*

3.9.3. Detection of Target proteins

Once the transfer was complete, the nitrocellulose membrane was placed in a tray with the side on which the proteins have been transferred facing the other; after washing the membrane with TBS-1X for 5', a sufficient quantity of Blocking Solution (10% BSA in TBS + 0.1% Tween-20) was added in order to have the membrane covered. This solution makes it possible to hinder the binding of immunoglobulins to non-specific sites and to reduce the detection of the signal of proteins other than those of interest. The membrane was immersed inside the Blocking solution while stirring for 60'. Subsequently, the Blocking Solution was replaced with a solution containing the primary antibody (diluted 1: 1000 and aimed at the proteins of interest) after carrying out 3 washes of 5' each with TBS + 0.1% Tween-20. Membrane was incubated at 4 ° C overnight, always under agitation. The next day, the membrane was subjected to 3 washes with TBS 0.1% + Tween-20 and subsequently incubated for 1h with the secondary antibody, (diluted 1: 2000) whose function is to recognize the primary antibody (**Fig. 17**) on the basis species specificity. At the end of the incubation, 3 washes were carried out with TBS 0.1% + Tween-20 in order to remove the excess of secondary antibody and the nitrocellulose membrane was further incubated for 5 minutes with a substrate of luminol and hydrogen peroxide in 1: 1 ratio and sensitive to peroxidase, an enzyme conjugated to the secondary antibody. The light emission that develops from the oxidation of luminol was detected by a luminometer and the image was acquired by Chemidoc which allows the display of the bands corresponding to the proteins of interest.

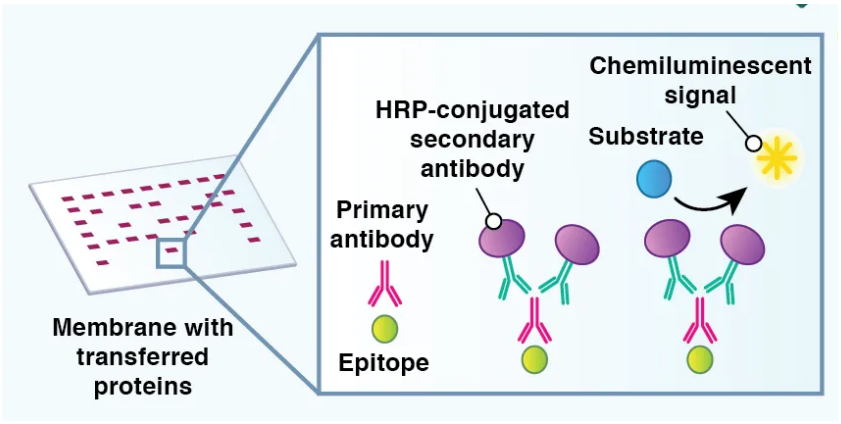


***Fig. 17** – Immunodetection*

3.10. BONE METABOLISM PROTEIN ARRAY

Human Bone Metabolism Antibody Array was used for quantitative measurement of 10 Human Bone Metabolism proteins: BMP-2, BMP-6, BMP-7, DKK-1, MMP-3, OPG, OPN, PDGF- BB, TGFβ3, TRANCE from cell lysates. Each glass slide (array) is spotted with 16 identical antibody arrays and is provided with a 16 well gasket to allow separate samples to be applied to each array (fig.18). Each antibody is spotted in quadruplicate on each array. For reproducible quantitation, eight of the arrays were used with a cocktail of protein standards to produce a standard curve.

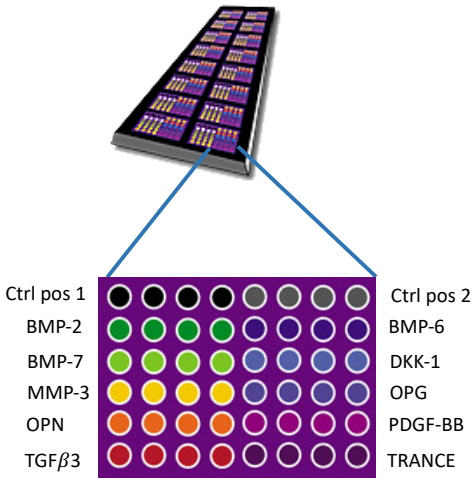


***Fig.18** – Representation of single array of 16 well glass side*

First, we prepared the reagents by diluting them to the right concentration; then we prepare the standard protein cocktail: starting from a stock solution we carried out 7 serial dilutions; finally, we prepared the samples to be analyzed: the protein extract was brought to a final concentration of 500 µg/mL using *sample diluent*.

After blocking the slides for 30' with *sample diluent*, the samples and standard solutions were aliquoted in the wells and incubated overnight at 4 °C, during this time the target proteins bind to

the specific antibodies immobilized on the slide surface (Fig.19 - 2). The next day we proceeded with 5 washes with *wash buffer I 1x* and 5 with *wash buffer II 1X,* as described by the protocol, and subsequently 80 µL of *detection antibody* were added to each well and incubated overnight at 4° C.

The *Detection antibody* is the biotinylated secondary antibody that binds to the antibody-antigen complex and is necessary for signal detection (**Fig.19 - 3**). Once this second incubation was completed and after carrying out the necessary washes, as described in the protocol, streptavidin conjugated to the fluorescent dye Cy3 (*Cy3 equivalentdye-conjugated streptavidin*) was added. In this way, each antigen-antibody complex is labeled with the Cy3 dye (**Fig.19 - 3**) and was detected by the axon GenePix laser scanner. The instrument has a 550 nm laser which excites the dye and captures the signal, returning an image in which each spot represents a specific target and the intensity of each spot is proportional to the number of antibody-antigen complexes formed and therefore it is proportional to the amount of target proteins present in the sample.

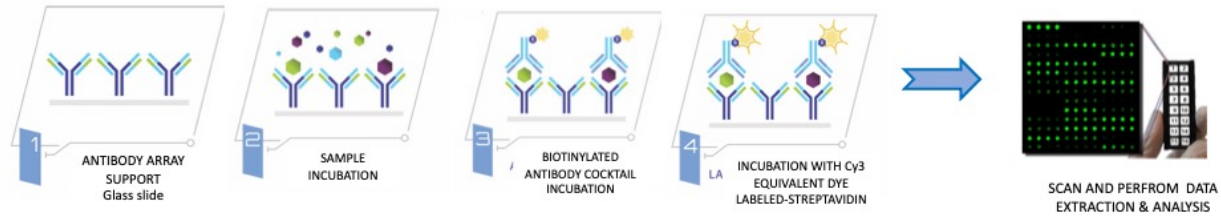


***Fig.19** – Procedure of Bone Metabolism Array*

3.11. REVERSE PHASE PROTEIN ARRAY (RPPA)

Reverse-Phase Protein microArrays (RPPA) is a technological platform for antibody-based (phospho)proteomic analysis. RPPA has been designed to study signal transduction pathways by relative quantification of proteins as well as their post-translational modifications.

In practice, an RPPA comprises a series of cell lysates immobilized on a nitrocellulose-coated slide. Then the molecule of interest is then detected, simultaneously in all the lysates, by means of a primary antibody / secondary antibody complex with an amplification system. From this particularity derives the name "Reverse" (Fig. 20).

In the RPPA each spot contains the cell lysate, which is usually in duplicate or tripled. Each array can contain hundreds of different samples, as well as calibrators and controls. Everything will be analyzed with a different antibody class for each slide and the signal will then be chemically amplified. The advantage of this technique is that it allows to measure hundreds of analytes simultaneously on hundreds of biological samples, with high sensitivity.

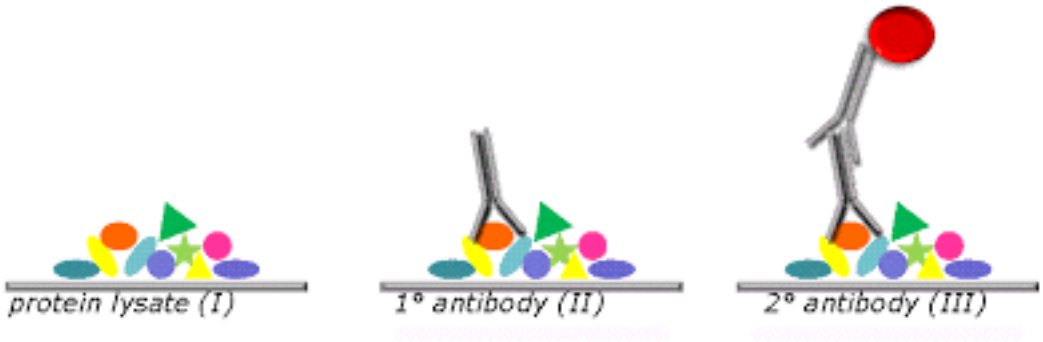


Fig.20 *– Representation of Reverse phase protein array*

3.11.1. Cell lysis and Protein Extraction

The optimal buffer for the extraction of proteins from tissue cells requires a detergent, a denaturing agent and a buffer. It includes:

• T-CEP (5%)

• 2X Tris-Glycine SDS Sample Buffer (45%)

• TPER (50%)

The lysates were solubilized in an adequate volume of Lysis Buffer and centrifuged for 15 'at 12,000 rpm and the supernatant was transferred to new eppendorf. The quantification of the protein extract was carried out using the Bradford method (described in par. 3.6). Finally, the lysates were brought to a concentration of 1 µg / µl and were heated to 100 ° C for complete denaturation of the proteins.

At this point, the samples can be stored at -80 ° C until the next step, the transfer of the samples to the nitrocellulose coated slide.

3.11.2. Protein deposition on support (Printing)

The Aushon 2470 arrayer instrument was used to print the samples on a nitrocellulose coated slide (Fig. 20)

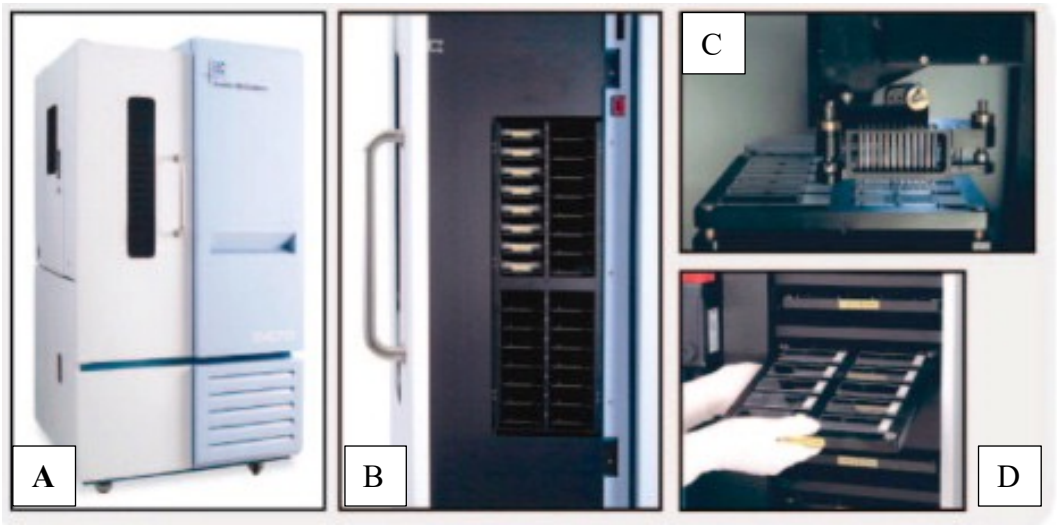


Fig. 20- *Various views of the Aushon BioSystems 2470 microarray*

Aushon 2470 is an automated platform that allows the deposition of samples on the nitrocellulose substrate accurately and precisely through "pins", which transfer a quantity of lysate in the order of the nanoliter, resulting in the formation of a spot. The pins are designed with soft-touch deposition technology and are located in a printing chamber isolated from the external environment (Fig. 20 C). The printing head moves only along the Z axis, while the plate moves along the X and Y axis. To print, the samples were loaded onto a 384-well plate, in a precise order, previously established, based on the desired final result.

The 40 slides coated with nitrocellulose membrane were placed on a special plate and numbered in ascending order (Fig. 20 D) considering. the number of antibodies (endpoints) to be analyzed and the slides for the evaluation of total proteins and negatives.

Calibrators, or positive controls, with scalar dilutions (A431+ EGF, A431+ Pervanadate, HeLa + Pervanadate, Jurkat + Calyculin, Jurkat+etoposide) were deposited in each slide.

Each sample was deposited 3 times in the same place and in triplicate.

3.11.3. Detection of proteins by Immunostaining

Before submitting the samples to immunostaining, the non-specific binding sites on the nitrocellulose membrane must be blocked.

The blocking takes place in a process divided into two main parts:

1. Antigen recovery using a medium strength stripping reagent (*Re-blot buffer*) for 15', followed by two washes in PBS.
2. Blocking of non-specific links with *I-Block* for at least one hour.

At this point the slides can be subjected to the staining process. Staining takes place with DAKO autostainer (Fig. 21).

Fig.21 *- DAKO autostainer*

This tool allows automatic processing of slides.

It is equipped with a program that allows the use of different protocols. A maximum of 36 slides can be processed during the same run. Individual slides can be programmed to receive different antibodies.

The program foresees that each slide is treated with:

1. Blockade of endogenous H_2O_2
2. Avidin for blocking endogenous biotin
3. Biotin for the blockade of endogenous avidin
4. Protein block is a ready-to-use reagent that blocks non-specific bonds on the nitrocellulose membrane
5. Selected primary antibody (endpoint)
6. Secondary antibody
7. SABC- streptavidin-biotin complex
8. Amplification reagent, responsible for signal amplification
9. LI-COR IRDye 680, used for signal detection

LI-COR IRDye 680 is a fluorophore that emits in the NIR range (near infrared-near infrared), with a maximum absorbance of 683 nm and a maximum emission of 710 nm.

The fluorescence method allows the operator to analyze very different signals present on the same slide.

For each immunostaining cycle there is a negative slide, processed exclusively with secondary antibody.

3.11.4. Determination of total protein

The normalization process of the intensity signal during data analysis is based on the total protein values. The evaluation of the possible variation of total proteins in the samples is important when considering the final volume of the lysates that are used for the slide deposition. In fact, since the volume is very small and considering that the number of arrays used can vary from 10 to 100, it is very common to see evaporation phenomena of the lysis buffer. This event therefore involves a variation of total proteins, which is not constant for each sample.

Every 10 printed slides, the tenth is dedicated to normalization.

Determining this variation allows the comparison of samples with different protein concentrations.

Total protein evaluation is performed by *Sypro Ruby blot stain* (Molecular Probes). It is a permanent coloring of proteins, detectable by fluorescence, with an excitation wavelength of 300 or 480 nm and an emission peak at 618 nm.

The color is composed of a ruthenium complex and is photostable over time.

Before staining, the slides were incubated with a *fixative solution* (3.5% acetic acid and 5% methanol, diluted in deionized water) for 15' on a rotating plate. Following 4 washes of 5 minutes each with deionized water, the slide was incubated with *Sypro Ruby blot stain* for a minimum of 30', protected from light, in order to avoid a reduction in signal intensity. After 1 minute washes with deionized water, the slide was dried away from light sources. The images were acquired using a laser scanner (Power Scanner^{TM}, Tecan) and the signal analysis was performed using the MicroVigene 5.999 software (Vigentech).

3.11.5. Determination of spot intensity

After staining with antibodies, the image of the slides was acquired with PowerScanner^{TM} (Tecan) and analyzed with the software MicroVigene 5.999 (Vigenetech), which identifies the single spots corresponding to the single samples, subtracts the value using the negative slide, averages the three replicates and normalizes each sample for the corresponding value verified by the total protein analysis.

3.12. MASS SPECTROMETRY

Mass Spectrometry was performed on conditioned media of C2C12 and LH cells and on total protein extract of HOB cells. Conditioned media were concentrated, quantified and added to Laemmli sample buffer (25 mM TrisHCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5% β-

mercaptoethanol, 0.0025% Bromophenol blue). For protein separation, equivalent amounts of extracts were resolved by SDS-PAGE on Mini- PROTEAN® TGXTM 4-15% Precast gels (Bio-Rad Laboratories); the resulting gels were stained overnight with Coomassie Brilliant Blue and the bands of interest were excised for Mass Spectrometry analysis.

Protein digestion was performed following a protocol reported in litera. Briefly, Coomassie-stained bands were diced into 1 mm3 cubes, destained for 30' with 50% acetonitrile in 100 mM ammonium bicarbonate buffer, dehydrated with 100% acetonitrile for 10 minutes, reduced with 10 mM DTT (1,4-Dithiothreitol, Sigma Aldrich) for 30 minutes at 56 °C and alkylated with 55 mM iodoacetamide (IAA, Sigma Aldrich) for 30 minutes in the dark. After alkylation, gel pieces were dehydrated with 100% acetonitrile and digested overnight with MS-grade trypsin (13 ng/µl in ammonium bicarbonate buffer 10nM with 10% acetonitrile; 90057, Pierce) at 37 °C. The digestion products were extracted with a solution of 5% formic acid and acetonitrile 1:2 for 15 minutes at 37°C in a shaker and were then lyophilized using a SpeedVac Concentrator (Savant).

Dry peptides from bands were resuspended in 40 µL of a mixture of water: acetonitrile: formic acid 97:3:2, sonicated for 10 min at room temperature and centrifuged at 12100 rcf for 10 min. Analyses were performed on an ESI Q Exactive Mass spectrometer (Thermo Scientific), controlled by Xcalibur (v. 29 build 2926) and interfaced with an Ultimate 3000 UHPLC pump.

The column (Zorbax SB-C18 RRHT, 2.1x50mm, 1.8 µ particle size, Agilent Technologies) was equilibrated with 0.3 ml/min of water 0.1% formic acid (A) with 2% acetonitrile (B); after sample injection (18 µl), B% was raised from 2→3%, then linearly increased from 3→21% in 19 min; B% was then brought to 90% in 4 min and kept at 90% B for three min, before the reconditioning step. The total runtime was 35 min.

ESI source was operated in positive mode; probe was heated at 290°C, capillary temperature was set at 270 °C; the following nitrogen flows (arbitrary units) were used to assist the ionization: Sheath ,36 Gas 40, Aux Gas 30, Sweep Gas 3; capillary voltage was set to 3.8 kV, S-Lens RF level was set at 45 (arbitrary units).

Profile MS and MS2 spectra were recorded from 200 to 2000 m/z in FULL MS/dd-MS2 (TOP5) mode, at a resolution of 70000 and 17500, respectively. The five most intense multi-charged ions were selected for MS2 nitrogen-promoted collision-induced dissociation (NCE=28). A precursor active exclusion of 20 seconds was set; peptide-like isotope pattern ions were preferred. The mass spectrometer was calibrated before the start of the analyses.

Raw data, converted into mascot generic format using MsConvert (v. 3.0.10730, ProteoWizard tools), were searched against Swiss-Prot (accessed June 2018; 16,977 sequences for Mus

musculus) for peptide sequences and C-RAP for contaminants with MASCOT (Version 2.4, Matrix Science).

Trypsin as proteolytic enzyme and carbamidomethyl cysteine as fixed modification were set in search parameters. Deamidated (NQ) and oxidated (M) were set as variable modifications. One missed cleavage was allowed. Mass tolerances were set at 10 ppm for the precursor ions and 0.05 Da for the product ions. Automatic decoy database search was used to estimate the false discovery rate, which was adjusted to £ 1%.

All proteins identified are listed in Table 5. GO annotation was used to identify protein clusters, this was obtained using Protein Analysis through Evolutionary Relationships (PANTHER version 14.1), STRING database (version 11.0) and the DAVID Bioinformatics Resources database (version 6.8).

3.13. STATISTICAL ANALYSIS

All data were analyzed using Prism version 6.0e (GraphPad Software). In vitro data are presented as the mean ± standard deviation (SD) from three independent experiments. Data for animal experiments are expressed as means ± standard deviation (SD). All statistical analyses were performed using one-way ANOVA followed by Dunnett's, Sidak's or Tukey's multiple-comparison tests. Differences between groups were considered statistically significant at $P < 0.05$.

4. Results

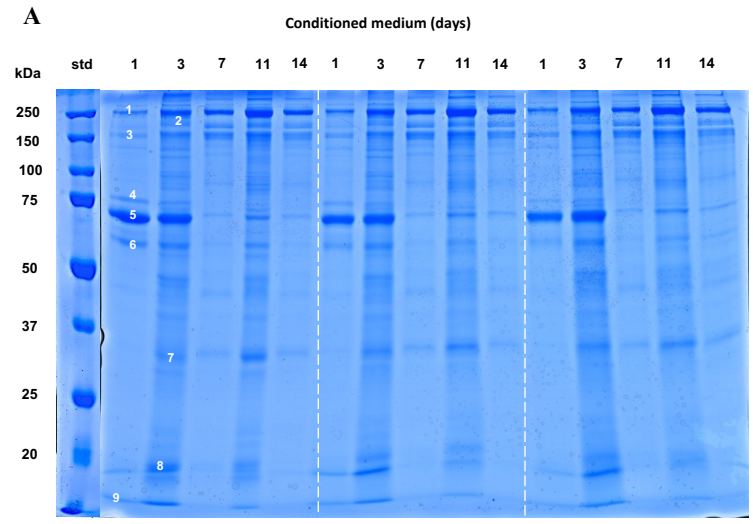
4.1. C2C12 SECRETOME ANALISYS BY MASS SPECTROMETRY

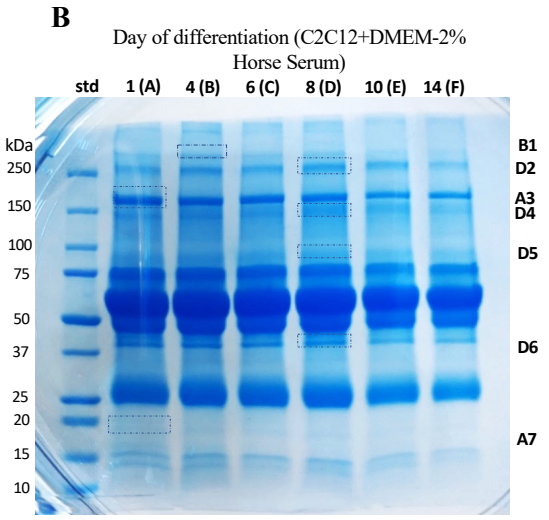
We performed a broad analysis of a partial secretome produced by murine C2C12 muscle cells and human LH muscle cells induced to differentiate, in order to find factors potentially involved in the modulation of osteogenic differentiation. Firstly, we performed three independent experiments of C2C12 differentiation with DMEM, 1% L-GLU, 1% P/S and 5 μg/ml Insulin, collecting the conditioned media (CM) on day 1, 3, 7, 11 and 14 after induction of the myogenic program. The CMs were then concentrated, separated by SDS-PAGE and the gels were stained with Coomassie Brilliant Blue. The Coomassie-stained gel revealed several bands differentially expressed during muscle cell differentiation, in a reproducible manner in the three trials (Fig. 22 A). Among these, we selected 9 bands that were more clearly visible and showed fluctuations in their intensity during the maturation process. These bands represent groups of proteins secreted by myoblasts/myotubes. More specifically, proteins 1, 3, 8 and 9 were secreted at every differentiation time point but with a fluctuating trend. Conversely, proteins 4, 5 and 6 were classified as "early" because they are present in the media collected from early stages of differentiation (day 1 and 3) and decrease or even disappear in later stages. Finally, proteins 2 and 7 were considered "late", since they are absent 24 hours after the induction of differentiation (day 1), and secreted later, from day 3 onwards, until the end of maturation (day 14), the earlier with an increasing trend and the latter with a fluctuating trend. Next, we performed Mass Spectrometry on two different media:

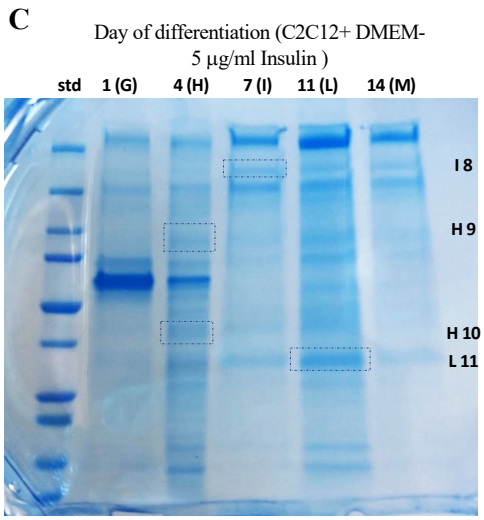
1) media obtained by C2C12 cells differentiated with two protocols: DMEM with 2% Horse serum and DMEM with 5 μg/ml insulin
2) media obtained by LH cells differentiated with DMEM with 2% Horse serum and 30 μg/ml insulin.

In C2C12 media we identified eleven bands of interest, according to the criterion of bands less intense and differentially expressed along maturation (FIG 22B- C). The bands highlighted with dashed rectangles were labeled with the sample code (A-F; G-M) and a progressive number (1-11), excised from the gel and processed for Mass Spectrometry analysis. In LH media we identified six bands of interest, in the late stage of differentiation (FIG 22D). The bands highlighted with dashed rectangles were labeled with the sample code (N-O) and a progressive number (12-17), excised from the gel and processed for Mass Spectrometry analysis. Through the Mass Spectrometry analysis, we found that every band contains several proteins with relative

abundances; all the proteins identified in [C2C12 DMEM-2% Horse Serum], [C2C12 DMEM-5 µg/ml Insulin] [C2C12 DMEM-2% Horse Serum - 30 µg/ml Insulin] differentiation protocols are listed in **table 5**, **table 6** and **table 7** respectively.







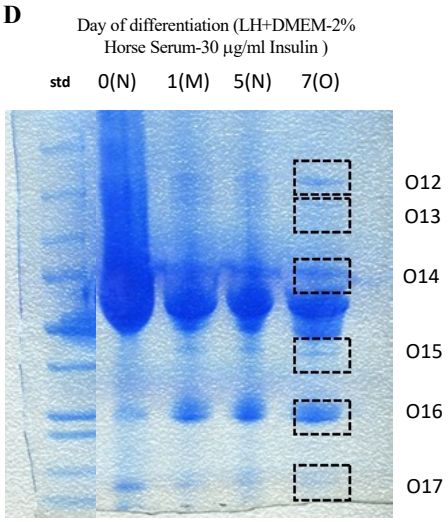


Fig.22 *– A) Coomassie-stained gel of the media conditioned by C2C12 cells in three independent experiments of differentiation.*
B-C) Representative scheme of the bands excised for Mass Spectrometry of C2C12+DMEM-2% Horse Serum and C2C12+ DMEM-5 μg/ml Insulin
D) Representative scheme of the bands excised for Mass Spectrometry of LH+DMEM-2% Horse Serum-30 μg/ml Insulin

Horse Serum			
ID Uniprot	**Protein name**	**Mass (kDa)**	**MASCOT Protein score**
FINC_MOUSE	Fibronectin	276,017	652
VIME_MOUSE	Vimentin	53,712	552
PZP_MOUSE	Pregnancy zone protein	167,116	273
CO1A2_MOUSE	Collagen alpha-2(I) chain	129,992	188
A2MG_MOUSE	Alpha-2-macroglobulin-P	165,674	186
CO3A1_MOUSE	Collagen alpha-1(III) chain	140,112	182
CATB_MOUSE	Cathepsin B	38,168	128
CO4B_MOUSE	Complement C4-B	194,447	123
ALBU_MOUSE	Serum albumin	70,7	119
GELS_MOUSE	Gelsolin	86,287	105
CERU_MOUSE	Ceruloplasmin	121,872	102
PGS2_MOUSE	Decorin	40,126	77
PGS1_MOUSE	Biglycan	42069	75
PZP_MOUSE	Pregnancy zone protein	167,116	65
ACTB_MOUSE	Actin, cytoplasmic 1	42,052	64
FINC_MOUSE	Fibronectin	276,017	63
SPRC_MOUSE	SPARC	35,283	62
HPT_MOUSE	Haptoglobin	39,241	60
A2MG_MOUSE	Alpha-2-macroglobulin-P	165,674	58
EAF6_MOUSE	Chromatin modification-related protein MEAF6	21,636	55
NID1_MOUSE	Nidogen-1	139,302	51
TIMP2_MOUSE	Metalloproteinase inhibitor 2	24,996	49
CFAH_MOUSE	Complement factor H	143,722	48
MYPT2_MOUSE	Protein phosphatase 1 regulatory subunit 12B	109,326	47
NOS2_MOUSE	Nitric oxide synthase, inducible	131,861	46
MIME_MOUSE	Mimecan	34,333	44
FSTL1_MOUSE	Follistatin-related protein 1	35,672	41
TETN_MOUSE	Tetranectin	22,642	39
ATLA1_MOUSE	Atlastin-1	63,736	31
CYTC_MOUSE	Cystatin-C	15,749	29
PPIC_MOUSE	Peptidyl-prolyl cis-trans isomerase C	22,894	25

Table 5- *List of the proteins secreted by C2C12 cells differentiated with DMEM-2% Horse serum and identified by MASCOT. Proteins have been ordered based on the MASCOT protein score.*

Insulin			
ID Uniprot	**Protein name**	**Mass (kDa)**	**MASCOT Protein score**
CO3A1_MOUSE	Collagen alpha-1(III) chain	140,112	1969
CO1A2_MOUSE	Collagen alpha-2(I) chain	129,992	1316
CO1A1_MOUSE	Collagen alpha-1(I) chain	138,974	501

LDHA_MOUSE	L-lactate dehydrogenase A chain	36,817	318
ANXA1_MOUSE	Annexin A1	38,995	307
ALDOA_MOUSE	Fructose-bisphosphate aldolase A	39,787	283
PGK1_MOUSE	Phosphoglycerate kinase 1	44,921	192
ANXA2_MOUSE	Annexin A2	38,937	168
CO3A1_MOUSE	Collagen alpha-1(III) chain	140,112	144
CO1A1_MOUSE	Collagen alpha-1(I) chain	138,974	140
ACTB_MOUSE	Actin, cytoplasmic 1	42,052	140
FPPS_MOUSE	Farnesyl pyrophosphate synthase	40,898	132
CS1A_MOUSE	Complement C1s-A subcomponent	78,347	89
SFRP2_MOUSE	Secreted frizzled-related protein 2	34,473	79
SPB6_MOUSE	Serpin B6	42,913	73
LEG3_MOUSE	Galectin-3	27,612	64
PGS2_MOUSE	Decorin	40,126	61
SAHH_MOUSE	Adenosyl homocysteinase	48,17	59
CAPG_MOUSE	Macrophage-capping protein	39,501	59
CO5A1_MOUSE	Collagen alpha-1(V) chain	184,248	59
LMNA_MOUSE	Prelamin-A/C	74,478	58
TERA_MOUSE	Transitional endoplasmic reticulum ATPase	89,95	57
CATB_MOUSE	Cathepsin B	38,168	56
HS90B_MOUSE	Heat shock protein HSP 90-beta	83,571	55
EF2_MOUSE	Elongation factor 2	96,222	52
SPRC_MOUSE	SPARC	35,283	52
HS90A_MOUSE	Heat shock protein HSP 90-alpha	85,134	51
SODE_MOUSE	Extracellular superoxide dismutase [Cu-Zn]	27,717	50
AATC_MOUSE	Aspartate aminotransferase, cytoplasmic	46,504	46
MSLN_MOUSE	Mesothelin	70,177	46
ANXA3_MOUSE	Annexin A3	36,533	46
FINC_MOUSE	Fibronectin	276,017	45
ILEUA_MOUSE	Leukocyte elastase inhibitor A	42,719	45
NID2_MOUSE	Nidogen-2	156,61	44
AEBP1_MOUSE	Adipocyte enhancer-binding protein 1	129,026	43
1433E_MOUSE	14-3-3 protein epsilon	29,326	40
IDHC_MOUSE	Isocitrate dehydrogenase [NADP] cytoplasmic	47,044	39
ROA2_MOUSE	Heterogeneous nuclear ribonucleoproteins A2/B1	37,437	38
TIMP1_MOUSE	Metalloproteinase inhibitor 1	23,298	37
EF1A1_MOUSE	Elongation factor 1-alpha 1	50,424	36
CATL1_MOUSE	Cathepsin L1	38,093	36
ANXA5_MOUSE	Annexin A5	35,787	35
FSTL1_MOUSE	Follistatin-related protein 1	35,672	34
ROAA_MOUSE	Heterogeneous nuclear ribonucleoprotein A/B	30,926	33
MDHC_MOUSE	Malate dehydrogenase, cytoplasmic	36,659	29
TPM1_MOUSE	Tropomyosin alpha-1 chain	32,718	27

FINC_MOUSE	Fibronectin	276,017	27
TSP1_MOUSE	Thrombospondin-1	133,555	26
GELS_MOUSE	Gelsolin	86,287	24
MYH9_MOUSE	Myosin-9	227,429	22
IBP7_MOUSE	Insulin-like growth factor -binding protein 7	29,977	21
COPD_MOUSE	Coatomer subunit delta	57,649	21
DOC11_MOUSE	Dedicator of cytokinesis protein 11	240,242	21
MDHM_MOUSE	Malate dehydrogenase, mitochondrial	36,045	21
ACTB_MOUSE	Actin, cytoplasmic 1	42,052	20
ANXA4_MOUSE	Annexin A4	36,178	19
BIG3_MOUSE	Brefeldin A-inhibited guanine nucleotide-exchange protein 3	243,245	17
PHAG1_MOUSE	Phosphoprotein associated with glycosphingolipid-enriched microdomains 1	47,147	16

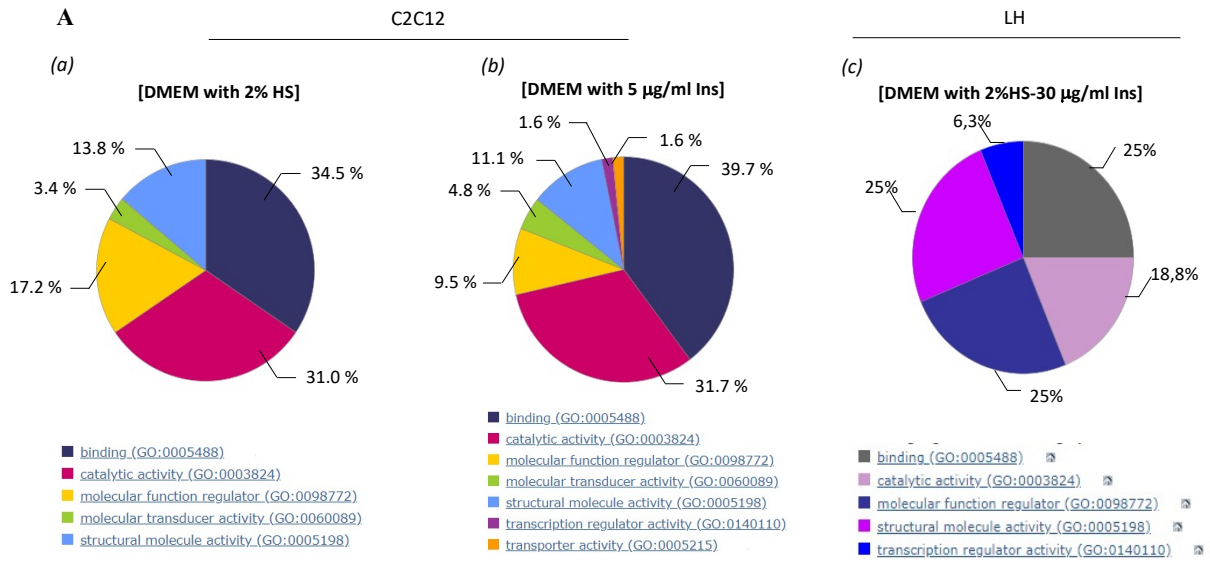
Table 6 - *List of the proteins secreted by C2C12 cells differentiated with DMEM-5 mg/ml insulin and identified by MASCOT. Proteins have been ordered based on the MASCOT protein score.*

LH+DMEM-2% Horse Serum-30 μg/ml Insulin			
ID Uniprot	**Protein Name**	**Mass (kDa)**	**MASCOT Protein score**
A2MG_HUMAN	Alpha-2-macroglobulin	164,613	340
A2MG_HUMAN	Alpha-2-macroglobulin	164,613	133
PZP_HUMAN	Pregnancy zone protein	165,242	120
HPT_HUMAN	Haptoglobin	45,861	103
ACTB_HUMAN	Actin, cytoplasmic 1	42,052	93
PZP_HUMAN	Pregnancy zone protein	165,242	81
CO4A_HUMAN	Complement C4-A	194,261	80
CO6A1_HUMAN	Collagen alpha-1(VI) chain	109,602	74
HPTR_HUMAN	Haptoglobin-related protein	39,518	58
CO4A_HUMAN	Complement C4-A	194,261	44
ALBU_HUMAN	Serum albumin	71,317	41
GELS_HUMAN	Gelsolin	86,043	39
CO3_HUMAN	Complement C3	188,569	37
LEG3_MOUSE	Galectin-3	27,612	37
THRB_HUMAN	Prothrombin	71,475	35
CO1A2_HUMAN	Collagen alpha-2(I)	129,749	34
CO1A1_HUMAN	Collagen alpha-1(I) chain	139,883	32
SPRC_HUMAN	SPARC	35,465	31
POSTN_HUMAN	Periostin	93,883	31

CO3_HUMAN	Complement C3	188,569	29
ALX4_HUMAN	Homeobox protein aristaless-like 4	44,613	28

Table 7 - *List of the proteins secreted by LH cells differentiated with DMEM-2% Horse Serum-30 µg/ml Insulin and identified by MASCOT. Proteins have been ordered based on the MASCOT protein score.*

All proteins were categorized in GO annotations analyzing molecular functions and biological processes. In Fig. 23A**,** it is clearly visible that the represented Gene Ontology categories are the same in the three procedures, except that in the Insulin protocol appear two additional functions ("transcription regulator activity" and "transporter activity"), though being represented by really few proteins (1.6% each). The majority of myogenic proteins found are involved in binding and catalytic activity. As for the biological processes, Fig. 23B shows that the proteins belong again to the same pathways, except for an additional process that appears only in the Insulin protocol: "developmental processes", though with only 1.4% total proteins. In both protocols, most of the proteins take part in cellular and metabolic processes, with a discrete fraction that is involved in the response to stimuli. Other represented activities are biological regulation, localization, and multicellular organismal processes. Of note, the expression of three proteins, above ones founded in C2C12 medium, were further analyzed for their interesting role during myogenesis and osteogenesis.



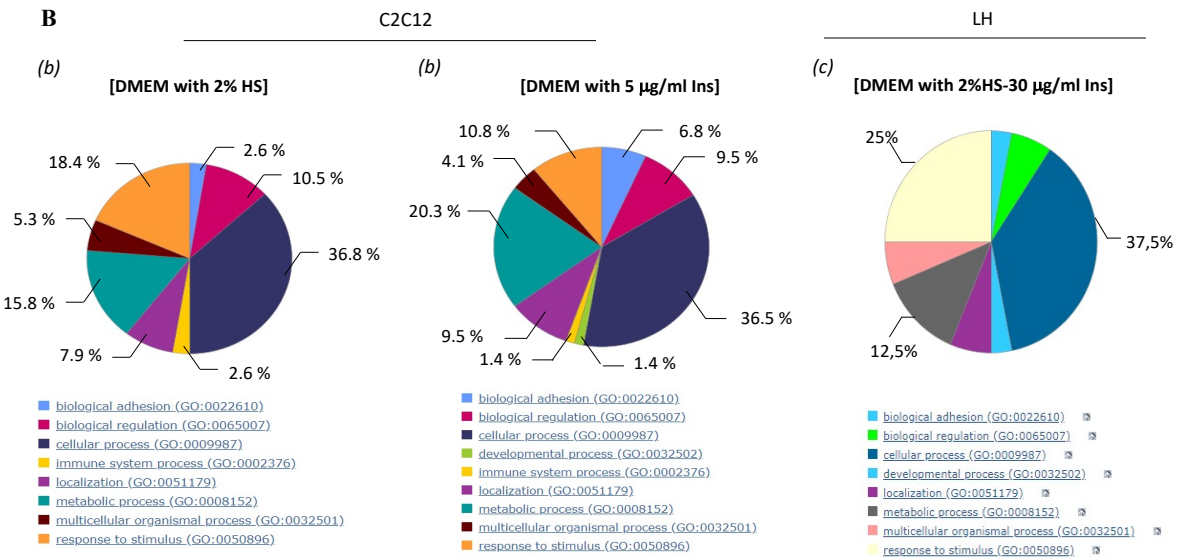


Fig. 23- *Enrichment analysis of the hits found by means of mass spectrometry (LCMSQE) of C2C12 (a-b) and LH (c). Myogenic proteins were clustered based on two PANTHER ontology classes: (A) molecular function and (B) biological process.*

Comparing the different proteins obtained we identified some interesting proteins that seem to have a regulatory function during bone processes: Osteoglycin, Decorin and Gal-3. In particular Gal-3 in both human and mouse muscle cells.

Gal-3, Osteoglycin and Decorin were investigated during myogenesis and osteogenesis by means of different cell lines: as myoblast cells we used mouse C2C12, mouse C57 and human LHCN-M2, while as osteoprogenitor cells, mouse MC3-T3, human hOB and human Mesenchymal Stem Cells were used. As expected from the literature, Gal- 3 has a prominent role for both the processes, myogenesis and osteogenesis, where it can be highlighted as a differentiation marker while others two proteins, Osteoglycin and Decorin, were stable along differentiation processes Fig. 24 (A - N).

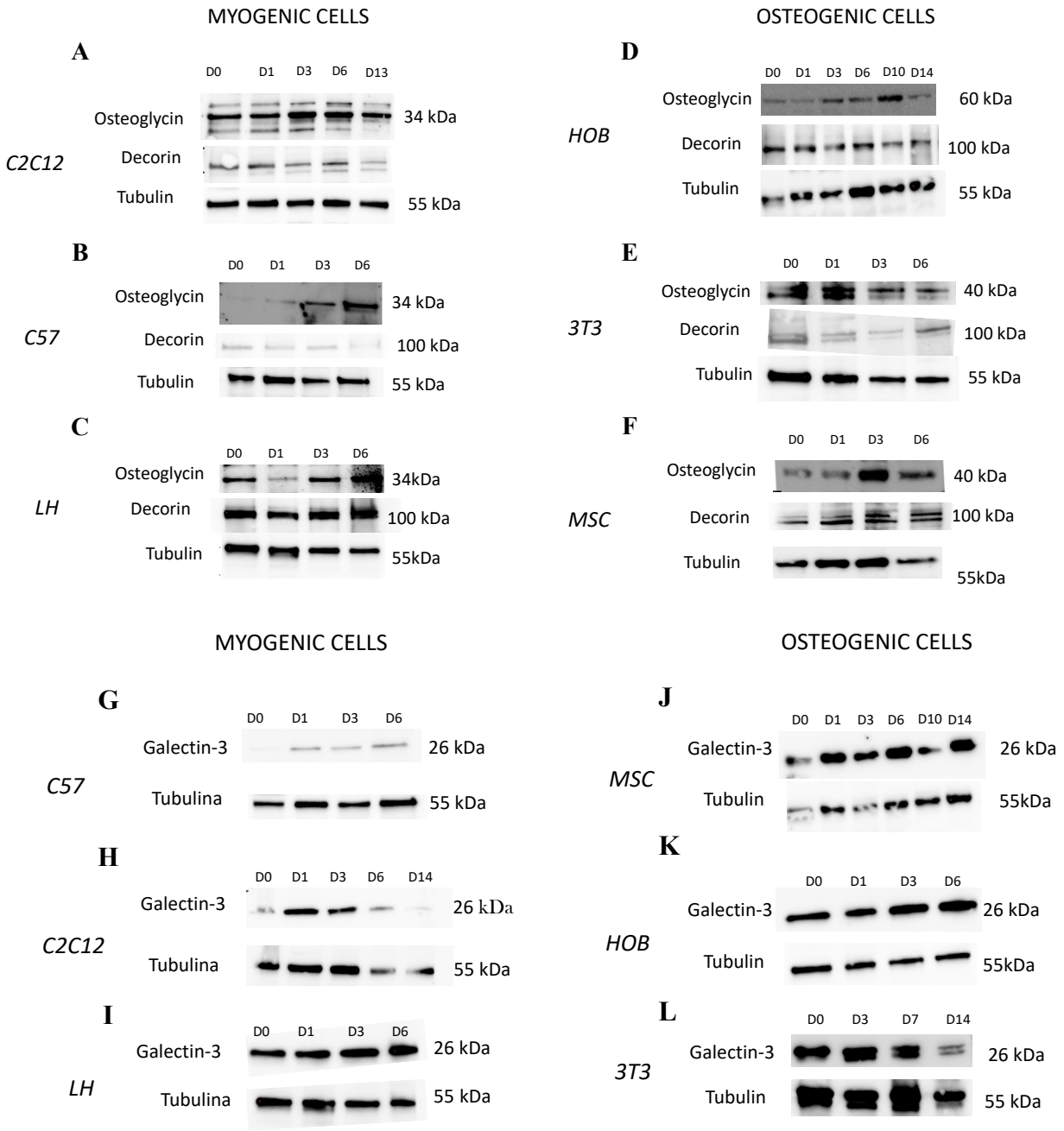


Fig. 24- *Expression of Osteoglycin and Decorin proteins (A-F) and Gal-3 protein (G-N) in cells of the myogenic and osteogenic lineage.*

For myogenic cells, we observed increased Gal-3 expression in early differentiating myoblasts. Nevertheless, the morphology of myotubes becomes much more evident in primary cell culture and the use of these kinds of cell culture (C57 and LHCN-M2) allowed us to obtain data at a closer physiological level than C2C12 cells approach would permit (Fig. 24 A-F). For osteogenesis process, our results corroborate these findings, well established, suggesting that Gal-3 may be a key player in all stages of bone biology, thus deserving to be thoroughly investigated as a potential target in bone disorders. In fact, we observed a net increase of Gal-3 during osteoblast differentiation, either for hOB, MC-3T3 and Human Mesenchymal Stem Cells (Fig. 24

G-L). Therefore, we focuse our experimental plan to the secreted form of Gal-3 that differs from intracellular one, from the structure and the functions. We confirmed the Mass Spectrometry data about the expression of Gal 3 in total lysates and cells supernatants by means of Western Blot showed in Fig. 25.

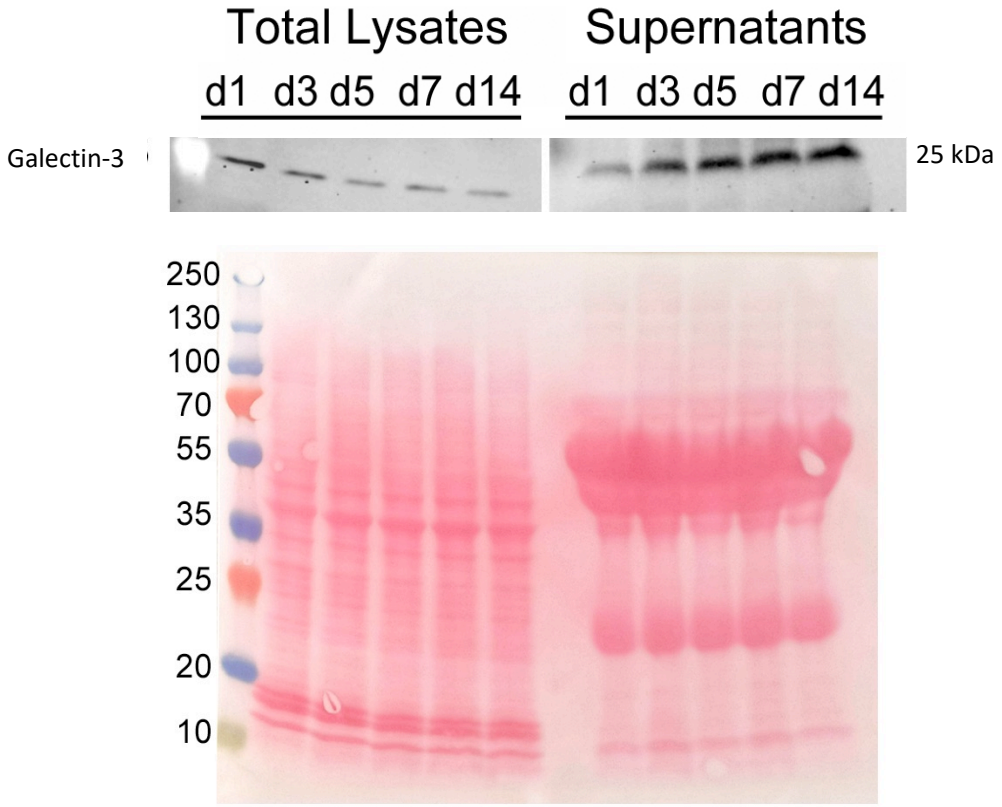


Fig. 25 *- Expression of Gal-3 protein in C2C12 supernatants*

4.2.GALECTIN-3 EXPRESSION IS STABLE DURING MOUSE GROWTH

To corroborate the production of Gal-3 by muscle cells *in vitro*, we moved to an *in vivo* context by analyzing the presence of Gal-3 in muscles of mice of different ages (young, 6 weeks; adult, 5 months; old, 18 months). For each age we isolated: i) a muscle composed of fast or glycolytic fibers (Gastrocnemius, GA); ii) a muscle composed of slow or oxidative fibers (Soleus, SO), which are subjected to phasic movement and important muscular work; iii) a muscle of the back (Trapezius, TR), less loaded according to the phasic activity of the limbs, to observe eventual relations between muscular work. Also, a muscle of the upper limb (Triceps Brachii, TB) was isolated, to compare it with the muscles of the lower limb.

In order to analyze the presence and the eventual amount of Gal-3 in muscles with different metabolic profiles, we performed Western Blot and ELISA assays. Two animals per age group were probed (n=2) and the same muscle was employed for both assays. The Gal-3 concentration

yielded by ELISA (ng/ml) was normalized on the muscle weight (ng/mg). The results obtained with Western Blot show that Gal-3 is synthesized by all the muscles analyzed and ~~and~~ it does not undergo significant changes during the growth of the mouse (Fig….). The ELISA assay showed a similar trend; Gal-3 values don't undergo significant statistical changes during the growth of the mouse ($p>0.05$) (Fig. 26).

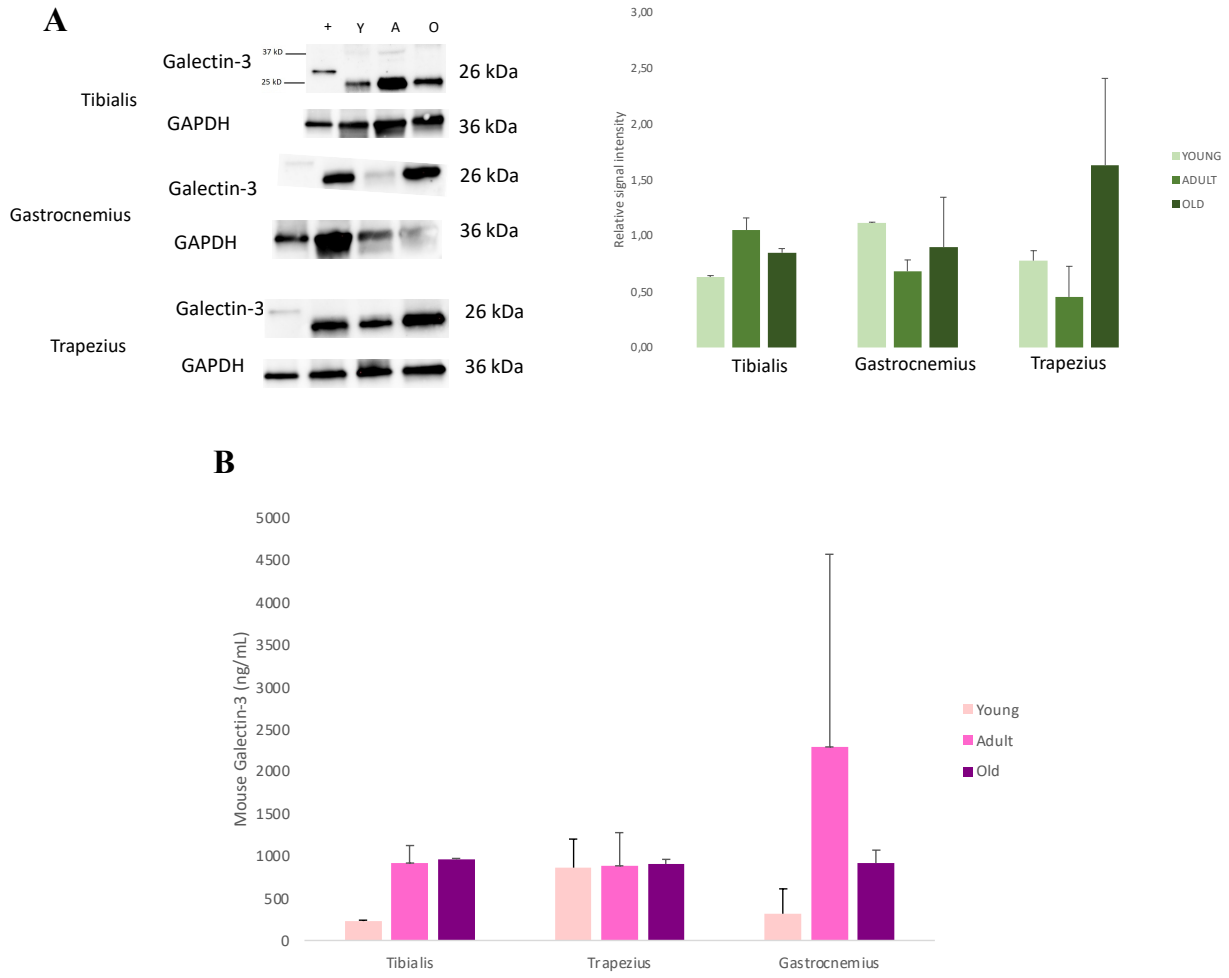
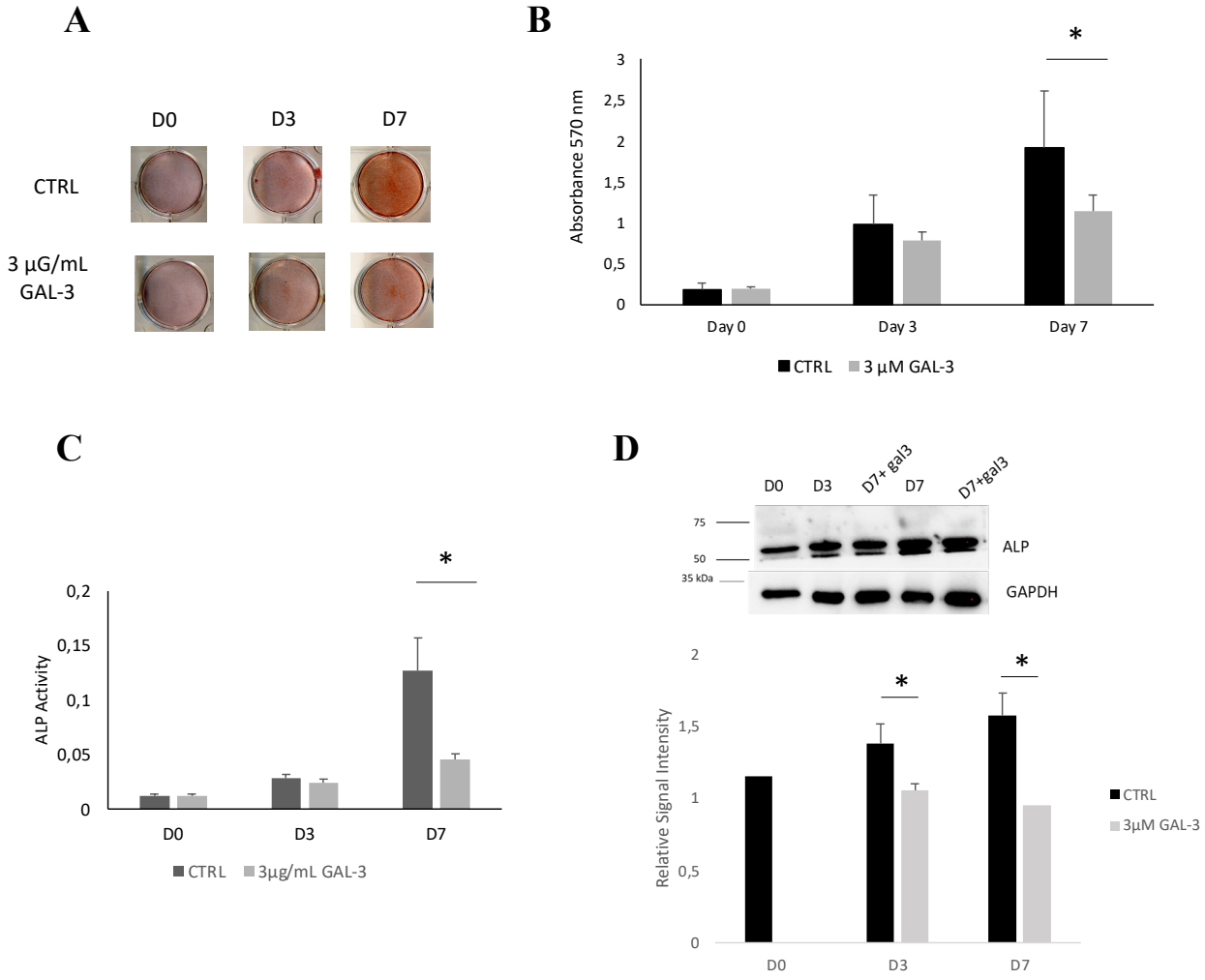


Fig. 26 - *A) Expression (WB) and B) Quantification (ELISA) of Gal-3 protein in three muscles of mice at three different ages.*
$p>0,05$.

4.3. SECRETED GALECTIN-3 CAN MODULATE FUNCTIONAL ACTIVITY OF OSTEOBLAST DIFFERENTIATION

Extracellular Gal-3 was investigated for its role during osteogenesis. We used recombinant Gal-3 (Fig. 27 A-E), to mimic the specific role of extracellular Gal-3, that is produced by muscle cells and acts bone cells. From our results, secreted Gal-3 was able to reduce osteoblast differentiation upon 7 days of osteogenic differentiation.

We examined whether the inhibitory effect of Gal-3 on osteoblast differentiation results from a transcriptional regulation of ALP or post-transcriptional regulation, such as inhibition of ALP enzyme activity. Western blot analysis revealed that the protein level of ALP remains stable in response to Gal-3 (Fig. 27 A-D), while it was potently inhibited the ALP enzyme activity. Calcium deposition, a marker for osteoblast differentiation, was detected by Alizarin Red stain. The number, size of particles, and total area were all reduced in response to Gal-3. Taken together, these findings demonstrate that Gal-3 impairs the early differentiation pathway of osteoblasts, consequently resulting in the inhibition of calcium deposition. Moreover, we analyzed the adhesion ability of differentiated cells, upon the stimulation with recombinant Gal-3. The ECM assay demonstrated that Gal-3, significantly decreases the cell adhesion to Collagen 1, Fibronectin, Laminin and Tenascin (Fig. 27E). These data corroborate the ability of Gal-3 to modulate the functional activity of osteoblast differentiation.



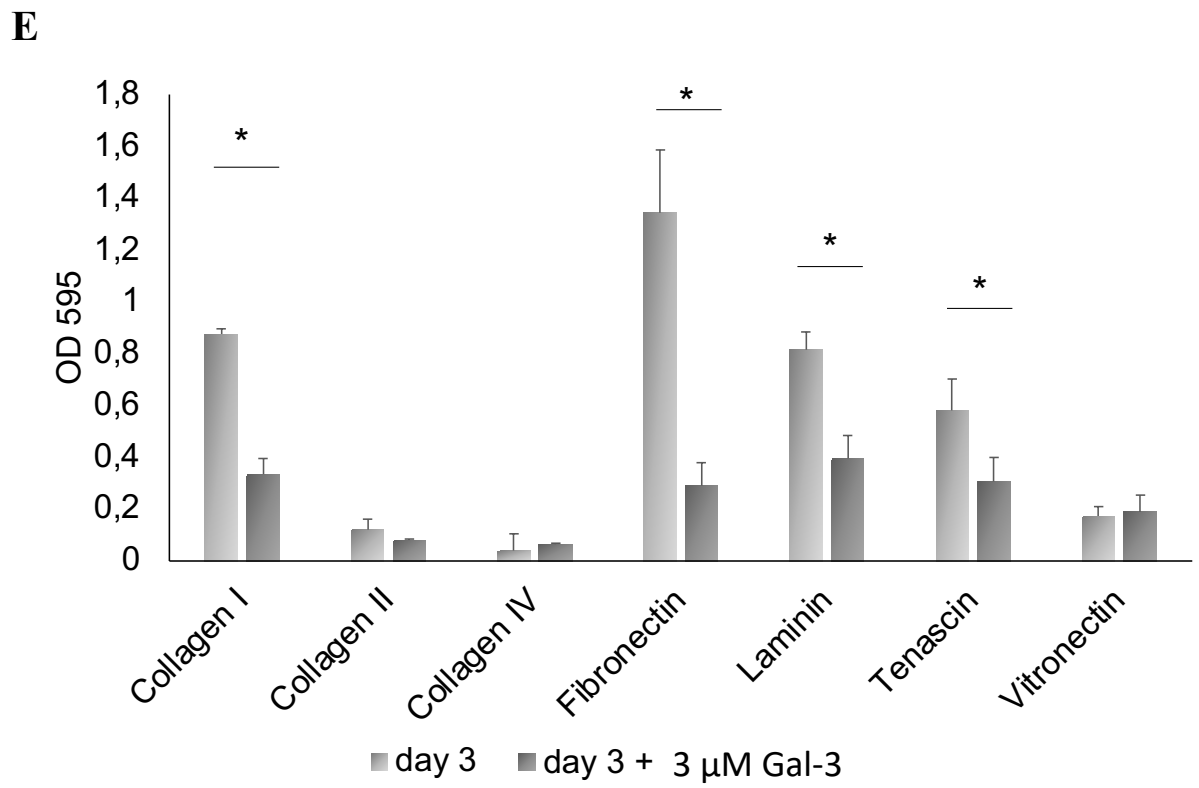


Fig. 27- *A) Representative images of cell monolayer staining with AR-S at the different experimental times Day 0 (D0), Day 3 (D3), Day 7 (D7) treated or not with 3µM Gal-3.*
B) Histogram that describes the trend of mineralization expressed as an absorbance value at 510 nm.
C) Histogram describing the ALP activity of 3T3 at different experimental times D0, D3, D7 treated or not with 3µM Gal-3
D) Expression of ALP in 3T3 at different experimental times D0, D3, D7 treated or not with 3µM Gal-3
E) Histogram describing the interaction of 3T3 treated or not with 3µM Gal -3 with molecules of the extracellular matrix.

*p< 0,05

4.4. MECHANISTIC INSIGHTS INTO SECRETED GALECTIN-3 FUNCTIONS DURING OSTEOGENIC DIFFERENTIATION

To elucidate the mechanism underlying the downregulation of osteogenesis induced by secreted Gal-3, we examined the whole proteome of MC3T3 stimulated with recombinant Gal-3, by Mass Spectrometry. In total, 117 proteins were identified in D0; 353 in D3; 366 in D3+ 3 µM Gal-3. Notably, the number of proteins expressed during the differentiation increases, of which 84 and 95 specifically expressed in D3 and D3+ gal respectively. Gene Ontology (GO) functional enrichment analysis performed with the identified total proteins of the two esperimental condition classifies the proteins based on their cellular location, the biological processes in which they are involved and their molecular functions showing those up or down regulated by the treatment (Fig. 28).

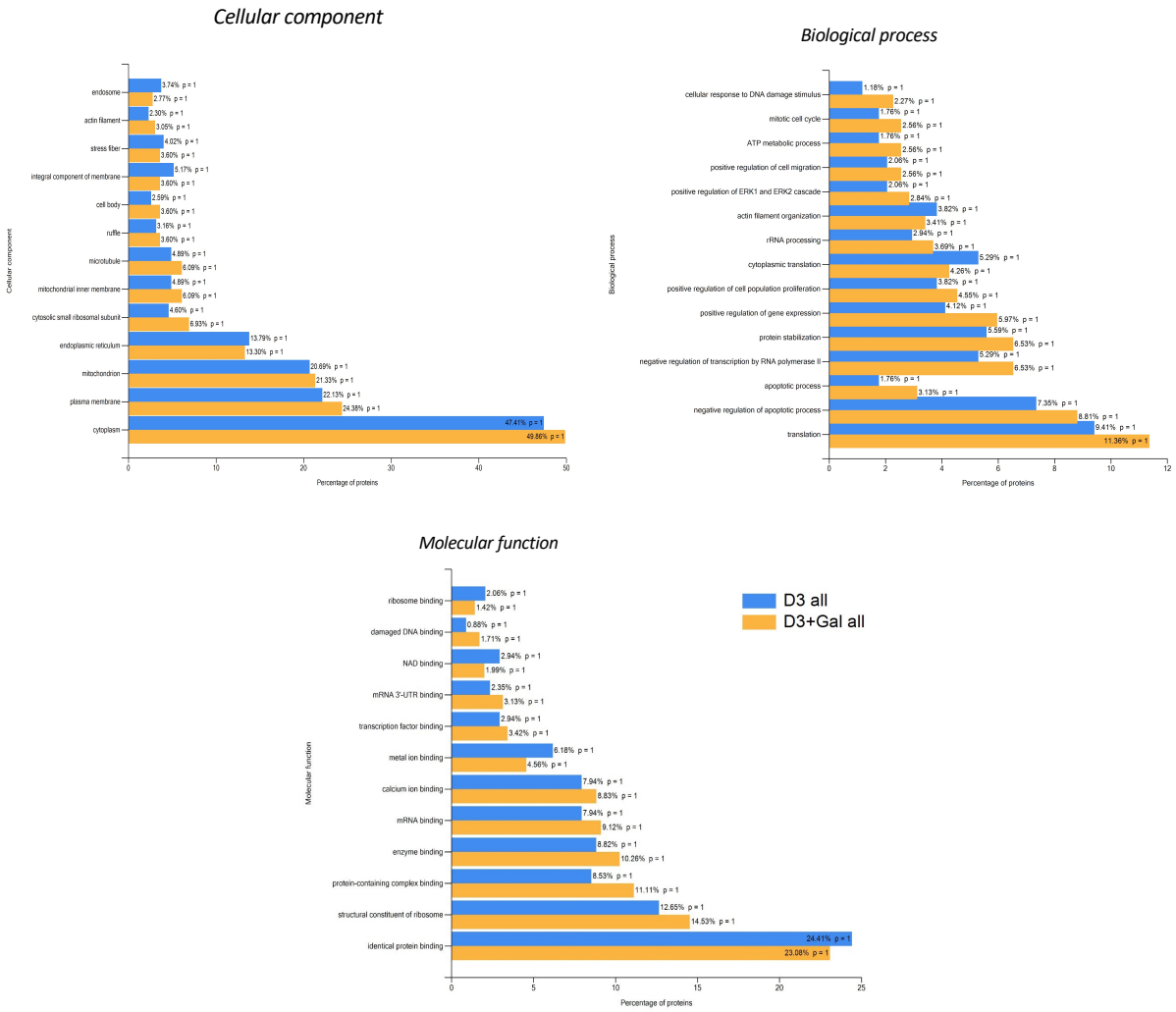


Fig. 28 - *Gene Ontology (GO) functional enrichment analysis performed with the identified total proteins of the two esperimental condition*

Subsequently quantitative analysis shows that some proteins are up or down regulated in presence of Gal-3, as shown in Fig. 29. These proteins are ribosomal proteins involved in translation processes, cytoskeleton proteins and signal proteins involved in pathway of cell metabolism.

A

		abs fold change	Cutoff 1.5 fold
Gene name	**Protein Name**	**D3Gal/D3**	**D3Gal/D3**
Rps19	40S ribosomal protein S19	2,240997774	↑
Rpl28	60S ribosomal protein L28	1,999659552	↑
Rpl4	60S ribosomal protein L4	1,885953421	↑
Rpl6	60S ribosomal protein L6	2,057982956	↑
Anxa1	Annexin A1	1,728316103	↑
Anxa2	Annexin A2	1,691555979	↑

Atp5f1a	ATP synthase subunit alpha, mitochondrial	1,657688788	↑
Calm1	Calmodulin-1	1,985773028	↑
Cd44	CD44 antigen	1,949668063	↑
Hspa5	Endoplasmic reticulum chaperone BiP	2,260484711	↑
Hsp90b1	Endoplasmin	2,118157896	↑
Hspa8	Heat shock cognate 71 kDa protein	2,118157896	↑
Hnrnpu	Heterogeneous nuclear ribonucleoprotein U	3,032816988	↑
Marcks	Myristoylated alanine-rich C-kinase substrate	2,109785731	↑
Lmna	Prelamin-A/C	1,612864091	↑
Ptma	Prothymosin alpha	3,656288076	↑
Cct4	T-complex protein 1 subunit delta	2,599557418	↑
Tpm1	Tropomyosin alpha-1 chain	5,325922515	↑
Tpm3	Tropomyosin alpha-3 chain	2,816187203	↑

B

		abs fold change	**Cutoff 1.5 fold**
Gene name	**Protein Name**	**D3Gal/D3**	**D3Gal/D3**
Rps3a	40S ribosomal protein S3a	1,854711321	↓
Rpl14	60S ribosomal protein L14	2,511739775	↓
Eif4b	Eukaryotic translation initiation factor 4B	1,731063899	↓
Capg	Macrophage-capping protein	1,608665442	↓
Ppia	Peptidyl-prolyl cis-trans isomerase A	2,061716554	↓
Pgam1	Phosphoglycerate mutase 1	1,567430993	↓
Sod1	Superoxide dismutase [Cu-Zn]	2,135518081	↓

Fig. 29- *A) List of proteins upregulated in presence of 3μg/mL Gal-3 in 3T3 cells after 3 days of treatment.*
B) List of proteins downregulated in presence of 3μg/mL Gal-3 in 3T3 cells after 3 days of treatment.

To corroborate the data obtained by Mass Spectrometry and deeper analyze, from a mechanical point of view, different signaling pathways activated upon Gal-3 stimulation or Gal-3 inhibition with TD139 (a competitive inhibitor of the active site of CRD domain,) we performed an RPPA assay using specific antibodies against multiple pathways: Akt/mTOR, RTKs and STATs.

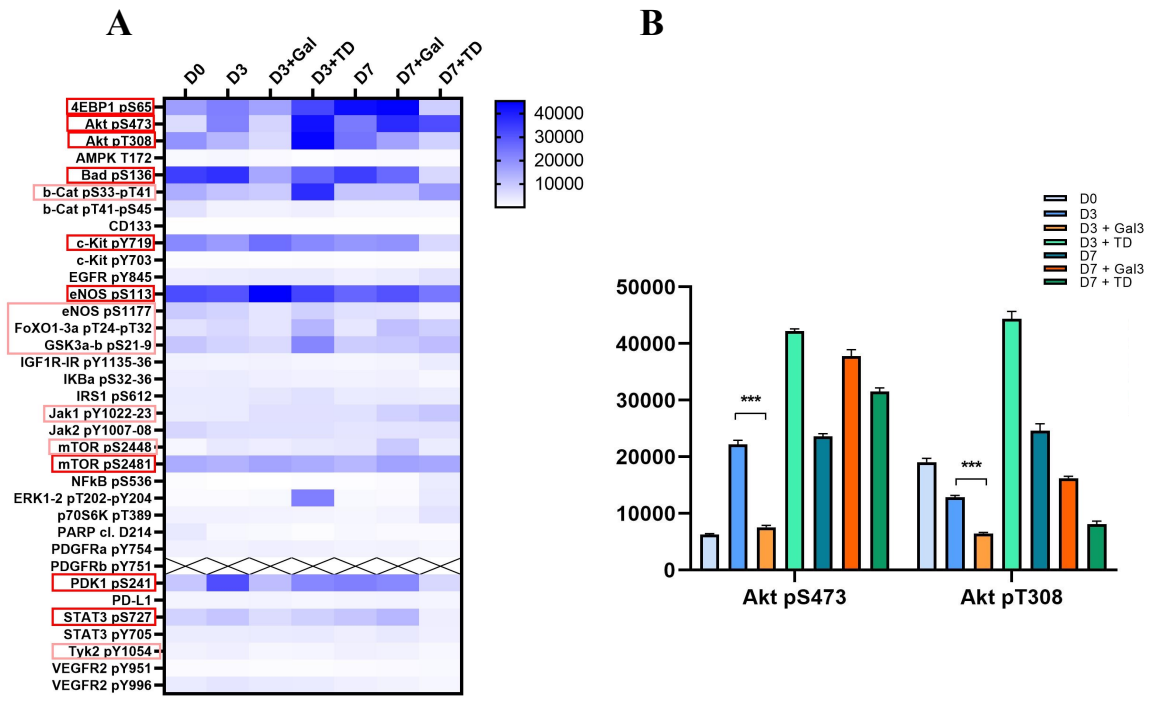
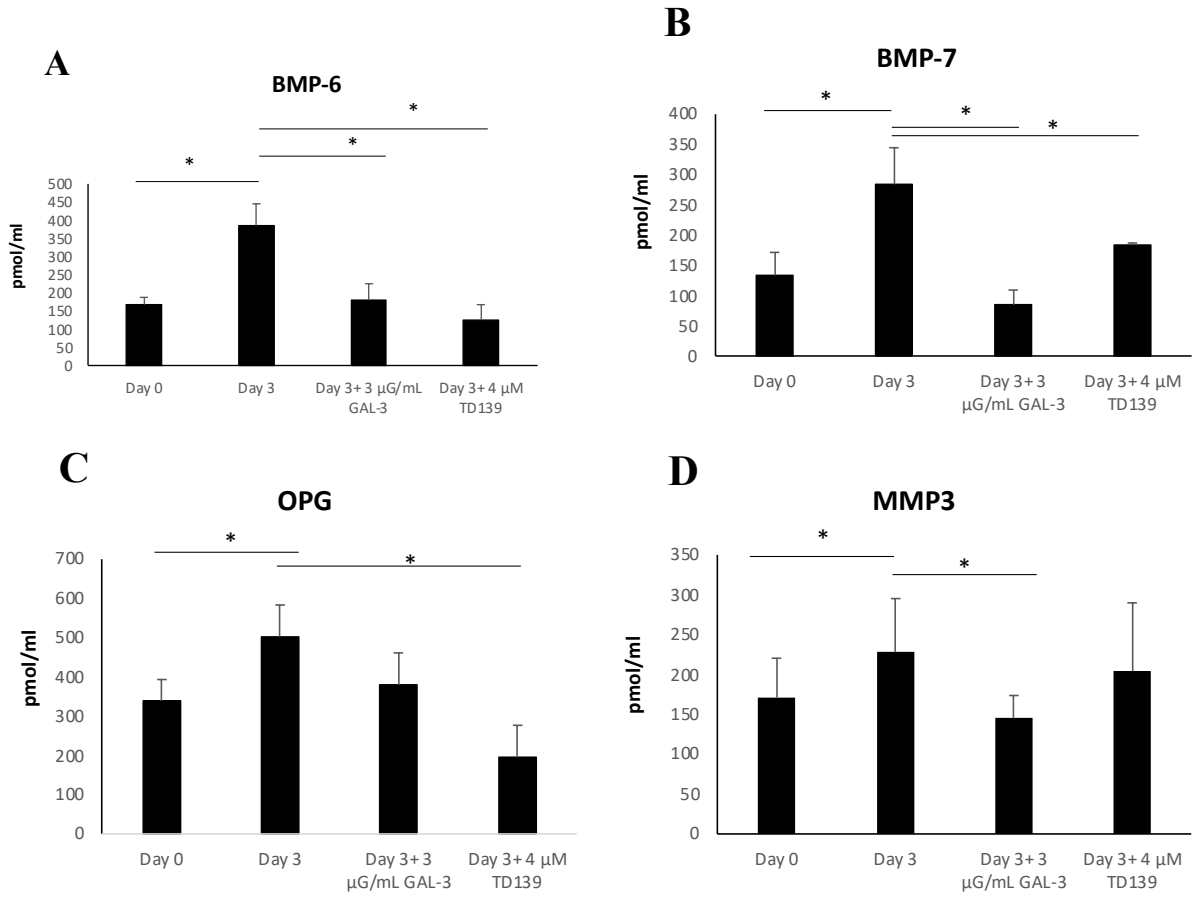


Fig. 30 *– A) Heat map of protein levels detected by RPPA. The biomarkers, a protein or a phosphorylated isotype detected with specific antibodies, are arrayed on the vertical axis, and the control and 3T3-treated samples are arrayed on the horizontal axis. The levels of each biomarker are identified with colors on a purple scale, from light (poorly expressed) to dark (highly expressed) B) Histogram that describes the expression of Akt phosphorylated (S473 and T308) in 3T3-treated sample.*
**** p<0,001*

The heat map shown in Fig. 30, demonstrated that Akt/mTor pathway (ser473-t308-pdk1-bad, foxo, gsk3, mTOR) is the regulatory loop relevantly activated during the osteogenic differentiation (monitored in both conditions EARLY OSTEOGENESIS, 3days and MIDDLE OSTEOGENESIS, 7 days). The addiction of Gal-3 can revert the activation of Akt/mTOR during the condition of EARLY osteogenic differentiation and in parallel, at the same time, is possible to see an opposite role mediated by the specific Gal-3 inhibitor, TD139 suggesting a specific role mediated by this extracellular component. In contrast with this result, it is of note that Akt/mTor pathway is completely activated upon the addiction of Gal-3 during the osteogenesis process monitored at 7 days of osteogenic induction, suggesting that this pathway is modulated by the extracellular protein, only in the EARLY phase of osteogenesis and subsequently in the MIDDLE phase, the regulatory mechanisms flattened. A similar behavior, reflecting the ability of Gal-3 to modulate signaling mechanism only during early phase of osteogenesis, was also highlighted for the Notch pathway (data not shown) that was confirmed by recent studies to be a negative regulatory mechanism downstream the Gal-3 role in decreasing the osteoblast functional activity. Bone morphogenetic proteins (BMPs) are secreted cytokines that control the fate and function of many different cell types. They were originally identified as factor(s) that induce the formation

of bone and cartilage when implanted at ectopic sites in rats. After binding to BMP ligands, homomeric dimers of the type II receptors form a tetrameric complex with homomeric dimers of the type I receptors and induce transphosphorylation of the type I receptors. This dynamic interaction leads to signal transduced through either Smads or MAPKs, which further activates the transcription of specific target genes involved in osteoblastic differentiation and bone formation. Since the results depicted here show that Gal-3 suppresses osteoblast differentiation, it was necessary to examine the possible relationship between Gal-3 and BMPs signaling. For each of our experimental set-up, we found that upon the addition of secreted Gal-3, there was a net decrease in the expression of BMP-7, BMP-6, Osteoprotegerin, MMP3 and RANKL (or TRANCE) (Fig. 31 A- E) proteins positively correlated with the osteoblast deposition. This is probably one of the most important mechanisms explaining the behavior of extracellular Gal-3 that negatively regulate the osteoblast fate and function.



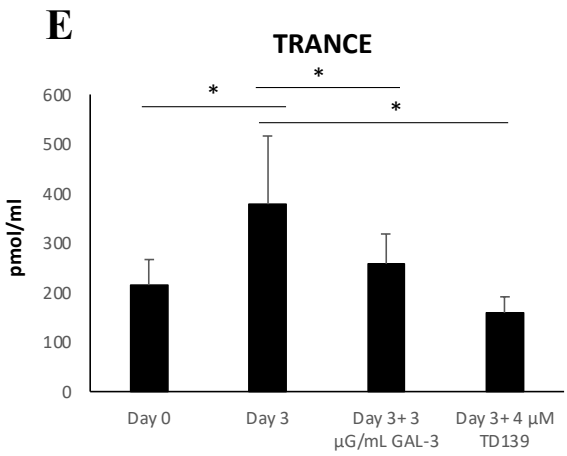


Fig. 31 -*Histograms of expression levels of BMP6, BMP7, OPG, MMP3, TRANCE proteins in 3T3-treated sample detected by Bone Metabolism Array.*
**p< 0,05*

5. Discussion

Galectin-3 (Gal-3) is a 29- to 35 kDa protein and it belongs to the family of the β- galactoside binding lectins. It is typically active when it is bound to its ligand and forms pentamers through its N-terminal domain (ND), while when its ligand is not present, it forms homodimer by self-association through its CRD domain. Gal-3 has several ligands, mainly glycoproteins, through which controls some signaling pathway [100]. It can act intracellularly and extracellularly affecting numerous cell function: cell proliferation and differentiation, apoptosis, mRNA maturation and cell adhesion. It is also a pleiotropic protein, and it is involved in many physiological and pathophysiological conditions[110]. As far as the bone tissue is concerned, its role is still not entirely clear. While Gal-3 appears to be an osteogenic marker and works as a pro-anabolic factor (in favor of the production of new bone mass)[131-132], on the other hand it seems to inhibit the formation of new bone [139]. This dual behavior could depend on the cellular localization of Gal-3, intracellular or extracellular. For this reason, the research work outlined in this thesis focuses on extracellular Gal-3, which acts as a paracrine factor. In fact, skeletal muscle is closely connected to the bone: they communicate both mechanically, through the contraction generated by the muscle that gives to bone the strength to move, and biochemically, through the production and secretion of various factors called myokines and osteokines, in a way called “Bone-muscle cross talk”.

The initial objective of our study was to identify new myokines that modulated osteogenesis. Using a proteomic approach based on mass spectrometry, several myokines were revealed in a medium obtained upon culture of human and mouse myotube. Comparing the different dataset obtained by Mass Spec analysis, we identified some interesting proteins that seem to have a regulatory function during bone processes: Osteoglycin, Decorin and Gal-3. The characterization of these three proteins during myogenesis and osteogenesis shows that Gal- 3 has a prominent role for both the processes identifying it as a differentiation marker while others two proteins, Osteoglycin and Decorin, were stable along differentiation processes. Osteoglycin and Decorin are two small leucin-rich proteoglycan, and they are already classified as Myokine that act on bone. Osteoglycin inhibits osteoblast differentiation of premature osteoblast but enhances osteoblast phenotype in well-differentiated osteoblasts probably through TGF-β-specific Smad 3/4 – responsive transcriptional activity. Decorin promote the formation of bone matrix and the deposit of Ca^{2+} to control the morphogenesis of the bone. Gal-3 has never before been classified as Myokine and its bone function is controversial and not completely understood. It seems to have a pro-anabolic action at the intracellular level but inhibits the formation of new bone when it acts at the extracellular level. Furthermore, being Gal-3 a differentiation marker, as mentioned above, it may be a key player in all

phases of bone biology, thus deserving to be deeply investigated as a potential target in bone disorders. For this reason, the subsequent purpose of the research work was to clarify the extracellular role of Gal-3 and define a possible mechanism of action.

Firstly, our experiments with the use of recombinant Gal-3 during osteogenic commitment of preosteoblast MC3T3, showed that the stimulus with Gal-3 reduces osteogenic differentiation, confirming its anti-anabolic extracellular action, in line with the results obtained by Nakajima et al[139]. This result is supported by functional assays that demonstrate a reduction in mineral matrix deposition, confirmed by Alizarin red assay, and a reduction of both ALP protein expression and activity in cells treated with Gal-3. Moreover, Gal-3 is able to modify the adhesion ability of differentiated cells upon the stimulation, significantly decreasing the cell adhesion to Collagen 1, Fibronectin, Laminin and Tenascin, thus suggesting the ability of Gal-3 to act as negative modulator of adhesion molecules expression, that in turn could be linked to the differentiation processes.

The central aim of the research work is to define the possible molecular mechanism of Gal- 3 involved in the reduction of osteogenic differentiation. First of all, Mass spectrometry analysis of the whole cellular proteome was performed to identify, for the first time, proteins specifically expressed in cells induced to differentiate in the presence of Gal-3 and to quantify those proteins whose expression change (up or down) upon Gal-3 administration. Notably, among the proteins modulated there are not proteins involved in osteogesis, but there are proteins involved in cell proliferation and translation processes, ribosomal proteins, cytoskeleton proteins and signal proteins involved in pathway of cell metabolism.

Under normal conditions, several signaling events essential to initiate bone-forming activity can be activated during osteogenic differentiation. In order to confirm the data obtained from the proteomic analysis, viz that the treatment with Gal-3 modulates the expression of some signal molecules, we analyzed the modulation of signal molecules involved in some of these pathways such as PI3k/Akt, WNT/β catenin and through the RRPA. The results demonstrate that Akt/mTor pathway is the regulatory loop relevantly activated during the osteogenic differentiation (monitored in both conditions EARLY OSTEOGENESIS and MIDDLE OSTEOGENESIS). The addition of Gal-3 can revert the activation of Akt/mTOR during the first three days of osteogenic differentiation and in parallel, at the same time, a specific Gal-3 inhibitor, TD139, has the opposite role suggesting a specific role mediated by this extracellular component. In contrast with this result, it is of note that Akt/mTor pathway is completely activated upon the addiction of Gal-3 during the osteogenesis process monitored at 7 days of osteogenic induction, suggesting that this pathway is modulated by the extracellular protein only in the EARLY phase of osteogenesis and subsequently, in the MIDDLE phase, the regulatory mechanisms flattened.

The activation of these signaling pathways leads to changes in bone metabolism. Therefore, using a specific protein array, we evaluated the principal protein involved in bone metabolism, including osteogenic marker such as Bone morphogenetic proteins (BMPs) that are secreted cytokines that control the fate and function of many different cell types. The dynamic interaction of BMP with its receptor leads to signal transduced through either Smads or MAPKs, which further activates the transcription of specific target genes involved in osteoblastic differentiation and bone formation. Here we found that the extracellular Gal-3 cause a net decrease in the expression of BMP-7, BMP-2, BMP-6, Osteoprotegerin (OPG) and RANKL (or TRANCE) proteins, positively correlated with the osteoblast deposition. This is probably one of the most important mechanism explaining the behavior of extracellular Gal-3 that negatively regulates the osteoblast fate and function.

Overall, the work presented in this PhD thesis project, contains important elements of originality and progress with respect to the state of the art:

we confirmed that extracellular Gal-3 has a negative effect on the production of a new bone mineral matrix, inhibiting osteoblast differentiation and modifying the cell adhesion ability of the cells.

for the first time, we analyzed the entire proteome of differentiating osteoblasts in presence of Gal-3 and identified the proteins mainly up or down regulated.

for the first time, we hypothesize a possible molecular mechanism of action of the extracellular Gal-3 on the bone tissue: Gal-3 inhibits PI3K/Akt/mTOR pathway, that is an essential pathway for osteoblast differentiation, probably preventing the ligand bond of target receptors (such as IGFR). Finally, this pathway regulates some gene expression including BMP and OPG that are osteogenic marker necessary in the early phase of osteogenic differentiation.

The next step of this work will be aimed to demonstrate that these results are linked to extracellular Gal-3 produced specifically by muscle cells. To this purpose, co-culture experiment between myobasts and osteoblasts are underway. We have planned to create a C2C12 stable line expressing Gal-3 and a Knockout line downregulating Gal-3, that will be put in direct contact with differentiating osteoblasts.

The final purpose of this study is to increase the knowledge about the mechanisms regulating the actions of extracellular Gal-3 the osteogenesis process. These new findings could be helpful for pave the way to a pharmacological approach for the treatment of muscle-skeletal diseases, especially in those pathological conditions (i.e. aging and inflammation) where there is a greater secretion of Gal-3 which negatively affects bone metabolism.

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7. Bibliography

1. Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S. Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. *The Journal of biological chemistry*. 2002;277(40):37377-37381. doi:10.1074/jbc.M206286200
2. Sottile J, Hocking DC. Fibronectin Polymerization Regulates the Composition and Stability of Extracellular Matrix Fibrils and Cell-Matrix Adhesions. *Molecular Biology of the Cell*. 2002;13(10):3546-3559. doi:10.1091/mbc.E02-01-0048
3. Nakamura-Ishizu A, Okuno Y, Omatsu Y, et al. Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. *Blood*. 2012;119(23):5429. doi:10.1182/BLOOD-2011-11-393645
4. Chen C, Jiang Z, Yang G. Laminins in osteogenic differentiation and pluripotency maintenance. *Differentiation*. 2020;114:13-19. doi:10.1016/J.DIFF.2020.05.002
5. Fisher LW, Fedarko NS. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. *Connective tissue research*. 2003;44 Suppl 1:33-40. Accessed January 6, 2018. http://www.ncbi.nlm.nih.gov/pubmed/12952171
6. Young MF. Bone matrix proteins: their function, regulation, and relationship to osteoporosis. *Osteoporosis International*. 2003;14(S3):35-42. doi:10.1007/s00198-002-1342-7
7. ElSayed SA, Bhimji SS. *Physiology, Bone*. StatPearls Publishing; 2017. Accessed January 7, 2018. http://www.ncbi.nlm.nih.gov/pubmed/28722997
8. Sommerfeldt DW, Rubin CT. Biology of bone and how it orchestrates the form and function of the skeleton. *European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society*. 2001;10 Suppl 2(0):S86-95. doi:10.1007/s005860100283
9. Rodan GA. Introduction to bone biology. *Bone*. 1992;13 Suppl 1:S3-6. Accessed January 11, 2018. http://www.ncbi.nlm.nih.gov/pubmed/1581117
10. Guntur AR, Rosen CJ. Bone as an endocrine organ. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists*. 2012;18(5):758-762. doi:10.4158/EP12141.RA
11. Panaroni C, Tzeng YS, Saeed H, Wu JY. Mesenchymal progenitors and the osteoblast lineage in bone marrow hematopoietic niches. *Current Osteoporosis Reports*. 2014;12(1):22-32. doi:10.1007/s11914-014-0190-7
12. Chen JH, Liu C, You L, Simmons CA. Boning up on Wolff's Law: Mechanical regulation of the cells that make and maintain bone. *Journal of Biomechanics*. 2010;43(1):108-118. doi:10.1016/j.jbiomech.2009.09.016
13. Arnett T. Regulation of bone cell function by acid-base balance. *The Proceedings of the Nutrition Society*. 2003;62(2):511-520. Accessed December 22, 2017. http://www.ncbi.nlm.nih.gov/pubmed/14506899
14. Lymperi S, Horwood N, Marley S, Gordon MY, Cope AP, Dazzi F. Strontium can increase some osteoblasts without increasing hematopoietic stem cells. *Blood*. 2008;111(3):1173-1181. doi:10.1182/blood-2007-03-082800
15. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 2003;425(6960):836-841. doi:10.1038/nature02041
16. Zaidi M. Skeletal remodeling in health and disease. *Nature Medicine*. 2007;13(7):791-801. doi:10.1038/nm1593
17. Wang H, Zhang P, Liu L, Zou L. Hierarchical organization and regulation of the hematopoietic stem cell osteoblastic niche. *Critical Reviews in Oncology/Hematology*. 2013;85(1):1-8. doi:10.1016/j.critrevonc.2012.05.004

18. Palumbo C, Ferretti M, Ardizzoni A, Zaffe D, Marotti G. Osteocyte-osteoclast morphological relationships and the putative role of osteocytes in bone remodeling. *J Musculoskel Neuron Interact*. 2001;1(4):327-332.
19. Bonewald LF. The amazing osteocyte. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2011;26(2):229-238. doi:10.1002/JBMR.320
20. Lee NK, Choi YG, Baik JY, et al. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood*. 2005;106(3):852-859. doi:10.1182/blood-2004-09-3662
21. Ono T, Nakashima T. Recent advances in osteoclast biology. *Histochemistry and cell biology*. 2018;149(4):325-341. doi:10.1007/S00418-018-1636-2
22. Edwards JR, Mundy GR. Advances in osteoclast biology: Old findings and new insights from mouse models. *Nature Reviews Rheumatology*. 2011;7(4):235-243. doi:10.1038/nrrheum.2011.23
23. Martin TJ, Seeman E. Bone remodelling: its local regulation and the emergence of bone fragility. *Best Practice and Research: Clinical Endocrinology and Metabolism*. 2008;22(5):701-722. doi:10.1016/j.beem.2008.07.006
24. Harada S ichi, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature*. 2003;423(6937):349-355. doi:10.1038/nature01660
25. Seeman E, Delmas PD. Bone quality--the material and structural basis of bone strength and fragility. *The New England journal of medicine*. 2006;354(21):2250-2261. doi:10.1056/NEJMra053077
26. Crane JL, Xian L, Cao X. Role of TGF-β Signaling in Coupling Bone Remodeling. *Methods in molecular biology (Clifton, NJ)*. 2016;1344:287-300. doi:10.1007/978-1-4939-2966-5_18
27. Koga T, Matsui Y, Asagiri M, et al. NFAT and Osterix cooperatively regulate bone formation. *Nature medicine*. 2005;11(8):880-885. doi:10.1038/nm1270
28. Maeda K, Kobayashi Y, Koide M, et al. The regulation of bone metabolism and disordersby wnt signaling. *International Journal of Molecular Sciences*. 2019;20(22). doi:10.3390/ijms20225525
29. Adami S, Bertoldo F, Brandi ML, et al. [Guidelines for the diagnosis, prevention and treatment of osteoporosis]. *Reumatismo*. 2009;61(4). doi:10.4081/REUMATISMO.2009.260
30. Riggs BL, Melton LJ. Involutional Osteoporosis. *New England Journal of Medicine*. 1986;314(26):1676-1686. doi:10.1056/NEJM198606263142605
31. Jilka RL. Molecular and cellular mechanisms of the anabolic effect of intermittent PTH. *Bone*. 2007;40(6):1434-1446. doi:10.1016/j.bone.2007.03.017
32. Almeida M, Han L, Martin-Millan M, et al. Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. *The Journal of biological chemistry*. 2007;282(37):27285-27297. doi:10.1074/jbc.M702810200
33. Kousteni S, Bellido T, Plotkin LI, et al. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell*. 2001;104(5):719-730. Accessed January 11, 2018. http://www.ncbi.nlm.nih.gov/pubmed/11257226
34. Nakamura T, Imai Y, Matsumoto T, et al. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell*. 2007;130(5):811-823. doi:10.1016/j.cell.2007.07.025
35. Weitzmann MN, Pacifici R. Estrogen deficiency and bone loss: an inflammatory tale. *Journal of Clinical Investigation*. 2006;116(5):1186-1194. doi:10.1172/JCI28550
36. (US) O of the SG. Diseases of Bone. Published online 2004. Accessed February 9, 2022. https://www.ncbi.nlm.nih.gov/books/NBK45506/
37. Frontera WR, Ochala J. Skeletal Muscle: A Brief Review of Structure and Function. doi:10.1007/s00223-014-9915-y

38. Wolfe RR. The underappreciated role of muscle in health and disease. *The American journal of clinical nutrition*. 2006;84(3):475-482. doi:10.1093/AJCN/84.3.475
39. Macaluso F, Myburgh • K H. Current evidence that exercise can increase the number of adult stem cells. doi:10.1007/s10974-012-9302-0
40. S. Hikida R. Aging changes in satellite cells and their functions. *Current aging science*. 2011;4(3):279-297. doi:10.2174/1874609811104030279
41. Bareja A, Holt JA, Luo G, et al. Human and Mouse Skeletal Muscle Stem Cells: Convergent and Divergent Mechanisms of Myogenesis. doi:10.1371/journal.pone.0090398
42. Mohr T, Andersen JL, Biering-Sørensen F, et al. Long-term adaptation to electrically induced cycle training in severe spinal cord injured individuals. *Spinal cord*. 1997;35(1):1-16. doi:10.1038/SJ.SC.3100343
43. Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, Pedersen BK. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *The Journal of physiology*. 2000;529 Pt 1(Pt 1):237-242. doi:10.1111/J.1469-7793.2000.00237.X
44. Pedersen BK, Steensberg A, Fischer C, et al. Searching for the exercise factor: is IL-6 a candidate? *Journal of muscle research and cell motility*. 2003;24(2-3):113-119. doi:10.1023/A:1026070911202
45. Whitham M, Parker BL, Friedrichsen M, et al. Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise. *Cell metabolism*. 2018;27(1):237-251.e4. doi:10.1016/J.CMET.2017.12.001
46. Severinsen MCK, Pedersen BK. Muscle–Organ Crosstalk: The Emerging Roles of Myokines. *Endocrine Reviews*. 2020;41(4):594. doi:10.1210/ENDREV/BNAA016
47. Sharma M, McFarlane C, Kambadur R, Kukreti H, Bonala S, Srinivasan S. Myostatin: expanding horizons. *IUBMB life*. 2015;67(8):589-600. doi:10.1002/IUB.1392
48. Anderson SB, Goldberg AL, Whitman M. Identification of a novel pool of extracellular pro-myostatin in skeletal muscle. *The Journal of biological chemistry*. 2008;283(11):7027-7035. doi:10.1074/JBC.M706678200
49. Scott Thies R, Chen T, Davies M v., et al. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. *Growth factors (Chur, Switzerland)*. 2001;18(4):251-259. doi:10.3109/08977190109029114
50. Han HQ, Zhou X, Mitch WE, Goldberg AL. Myostatin/activin pathway antagonism: molecular basis and therapeutic potential. *The international journal of biochemistry & cell biology*. 2013;45(10):2333-2347. doi:10.1016/J.BIOCEL.2013.05.019
51. Allen DL, Hittel DS, McPherron AC. Expression and function of myostatin in obesity, diabetes, and exercise adaptation. *Medicine and science in sports and exercise*. 2011;43(10):1828-1835. doi:10.1249/MSS.0B013E3182178BB4
52. Hittel DS, Berggren JR, Shearer J, Boyle K, Houmard JA. Increased secretion and expression of myostatin in skeletal muscle from extremely obese women. *Diabetes*. 2009;58(1):30-38. doi:10.2337/DB08-0943
53. Palsgaard J, Brøns C, Friedrichsen M, et al. Gene expression in skeletal muscle biopsies from people with type 2 diabetes and relatives: differential regulation of insulin signaling pathways. *PloS one*. 2009;4(8). doi:10.1371/JOURNAL.PONE.0006575
54. Smith RC, Lin BK. Myostatin inhibitors as therapies for muscle wasting associated with cancer and other disorders. *Current opinion in supportive and palliative care*. 2013;7(4):352-360. doi:10.1097/SPC.0000000000000013
55. Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, Pedersen BK. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *The Journal of physiology*. 2000;529 Pt 1(Pt 1):237-242. doi:10.1111/J.1469-7793.2000.00237.X

56. Bruunsgaard H, Galbo H, Halkjaer-Kristensen J, Johansen TL, MacLean DA, Pedersen BK. Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. *The Journal of physiology*. 1997;499 (Pt 3)(Pt 3):833-841. doi:10.1113/JPHYSIOL.1997.SP021972
57. FEBBRAIO MA, PEDERSEN BK. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2002;16(11):1335-1347. doi:10.1096/FJ.01-0876REV
58. MacIntyre DL, Sorichter S, Mair J, Berg A, McKenzie DC. Markers of inflammation and myofibrillar proteins following eccentric exercise in humans. *European journal of applied physiology*. 2001;84(3):180-186. doi:10.1007/S004210170002
59. Wolsk E, Mygind H, Grøndahl TS, Pedersen BK, van Hall G. IL-6 selectively stimulates fat metabolism in human skeletal muscle. *American journal of physiology Endocrinology and metabolism*. 2010;299(5). doi:10.1152/AJPENDO.00328.2010
60. Pal M, Febbraio MA, Whitham M. From cytokine to myokine: the emerging role of interleukin-6 in metabolic regulation. *Immunology and cell biology*. 2014;92(4):331-339. doi:10.1038/ICB.2014.16
61. Carey AL, Steinberg GR, Macaulay SL, et al. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. *Diabetes*. 2006;55(10):2688-2697. doi:10.2337/DB05-1404
62. van Hall G, Steensberg A, Sacchetti M, et al. Interleukin-6 stimulates lipolysis and fat oxidation in humans. *The Journal of clinical endocrinology and metabolism*. 2003;88(7):3005-3010. doi:10.1210/JC.2002-021687
63. Pedersen BK. Muscles and their myokines. *The Journal of experimental biology*. 2011;214(Pt 2):337-346. doi:10.1242/JEB.048074
64. Quinn LBS, Anderson BG, Drivdahl RH, Alvarez B, Argilés JM. Overexpression of interleukin-15 induces skeletal muscle hypertrophy in vitro: implications for treatment of muscle wasting disorders. *Experimental cell research*. 2002;280(1):55-63. doi:10.1006/EXCR.2002.5624
65. Pistilli EE, Alway SE. Systemic elevation of interleukin-15 in vivo promotes apoptosis in skeletal muscles of young adult and aged rats. *Biochemical and biophysical research communications*. 2008;373(1):20-24. doi:10.1016/J.BBRC.2008.05.188
66. López-Soriano J, Carbó N, Almendro V, et al. Rat liver lipogenesis is modulated by interleukin-15. *International journal of molecular medicine*. 2004;13(6):817-819. doi:10.3892/IJMM.13.6.817/HTML
67. Almendro V, Busquets S, Ametller E, et al. Effects of interleukin-15 on lipid oxidation: disposal of an oral [(14)C]-triolein load. *Biochimica et biophysica acta*. 2006;1761(1):37-42. doi:10.1016/J.BBALIP.2005.12.006
68. Mucci P, Durand F, Lebel B, Bousquet J, Préfaut C. Interleukins 1-beta, -8, and histamine increases in highly trained, exercising athletes. *Medicine and science in sports and exercise*. 2000;32(6):1094-1100. doi:10.1097/00005768-200006000-00009
69. Nieman DC, Henson DA, Smith LL, et al. Cytokine changes after a marathon race. *Journal of applied physiology (Bethesda, Md : 1985)*. 2001;91(1):109-114. doi:10.1152/JAPPL.2001.91.1.109
70. Kim GY, Lee JW, Ryu HC, Wei JD, Seong CM, Kim JH. Proinflammatory cytokine IL-1beta stimulates IL-8 synthesis in mast cells via a leukotriene B4 receptor 2-linked pathway, contributing to angiogenesis. *Journal of immunology (Baltimore, Md : 1950)*. 2010;184(7):3946-3954. doi:10.4049/JIMMUNOL.0901735
71. Boström P, Wu J, Jedrychowski MP, et al. A PGC1α-dependent myokine that drives browning of white fat and thermogenesis. *Nature*. 2012;481(7382):463. doi:10.1038/NATURE10777

72. Lee P, Linderman JD, Smith S, et al. Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell metabolism*. 2014;19(2):302. doi:10.1016/J.CMET.2013.12.017
73. Wu J, Boström P, Sparks LM, et al. Beige Adipocytes are a Distinct Type of Thermogenic Fat Cell in Mouse and Human. *Cell*. 2012;150(2):366. doi:10.1016/J.CELL.2012.05.016
74. Boström P, Wu J, Jedrychowski MP, et al. A PGC1α-dependent myokine that drives browning of white fat and thermogenesis. *Nature*. 2012;481(7382):463. doi:10.1038/NATURE10777
75. Vaughan RA, Gannon NP, Barberena MA, et al. Characterization of the metabolic effects of irisin on skeletal muscle in vitro. *Diabetes, Obesity and Metabolism*. 2014;16(8):711-718. doi:10.1111/DOM.12268
76. Sanchis-Gomar F, Lippi G, Mayero S, Perez-Quilis C, García-Giménez JL. Irisin: a new potential hormonal target for the treatment of obesity and type 2 diabetes. *Journal of diabetes*. 2012;4(3):196-196. doi:10.1111/J.1753-0407.2012.00194.X
77. Li G bin, Zhang L, Wang DE, et al. Muscle-bone crosstalk and potential therapies for sarco-osteoporosis. *Journal of cellular biochemistry*. 2019;120(9):14262-14273. doi:10.1002/JCB.28946
78. Karsenty G, Ferron M. The contribution of bone to whole-organism physiology. *Nature*. 2012;481(7381):314-320. doi:10.1038/NATURE10763
79. Frost HM. Bone's mechanostat: a 2003 update. *The anatomical record Part A, Discoveries in molecular, cellular, and evolutionary biology*. 2003;275(2):1081-1101. doi:10.1002/AR.A.10119
80. Germiller JA, Goldstein SA. Structure and function of embryonic growth plate in the absence of functioning skeletal muscle. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*. 1997;15(3):362-370. doi:10.1002/JOR.1100150308
81. Nowlan NC, Murphy P, Prendergast PJ. A dynamic pattern of mechanical stimulation promotes ossification in avian embryonic long bones. *Journal of biomechanics*. 2008;41(2):249-258. doi:10.1016/J.JBIOMECH.2007.09.031
82. Roddy KA, Prendergast PJ, Murphy P. Mechanical influences on morphogenesis of the knee joint revealed through morphological, molecular and computational analysis of immobilised embryos. *PloS one*. 2011;6(2). doi:10.1371/JOURNAL.PONE.0017526
83. Dankbar B, Fennen M, Brunert D, et al. Myostatin is a direct regulator of osteoclast differentiation and its inhibition reduces inflammatory joint destruction in mice. *Nature medicine*. 2015;21(9):1085-1090. doi:10.1038/NM.3917
84. Bialek P, Parkington J, Li X, et al. A myostatin and activin decoy receptor enhances bone formation in mice. *Bone*. 2014;60:162-171. doi:10.1016/J.BONE.2013.12.002
85. Hamrick MW, Shi X, Zhang W, et al. Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone*. 2007;40(6):1544-1553. doi:10.1016/J.BONE.2007.02.012
86. Chen YS, Guo Q, Guo LJ, et al. GDF8 inhibits bone formation and promotes bone resorption in mice. *Clinical and experimental pharmacology & physiology*. 2017;44(4):500-508. doi:10.1111/1440-1681.12728
87. Hamrick MW, McNeil PL, Patterson SL. Role of muscle-derived growth factors in bone formation. *Journal of musculoskeletal & neuronal interactions*. 2010;10(1):64-70. Accessed January 16, 2022. https://pubmed.ncbi.nlm.nih.gov/20190381/
88. Guntur AR, Rosen CJ. IGF-1 regulation of key signaling pathways in bone. *BoneKEy reports*. 2013;2. doi:10.1038/BONEKEY.2013.171
89. Niu T, Rosen CJ. The insulin-like growth factor-I gene and osteoporosis: a critical appraisal. *Gene*. 2005;361(1-2):38-56. doi:10.1016/J.GENE.2005.07.016
90. Douglas Coffin J, Homer-Bouthiette C, Hurley MM. Fibroblast Growth Factor 2 and Its Receptors in Bone Biology and Disease. *Journal of the Endocrine Society*. 2018;2(7):657-671. doi:10.1210/JS.2018-00105

91. Colaianni G, Cuscito C, Mongelli T, et al. Irisin enhances osteoblast differentiation in vitro. *International journal of endocrinology*. 2014;2014. doi:10.1155/2014/902186
92. Colaianni G, Cuscito C, Mongelli T, et al. The myokine irisin increases cortical bone mass. *Proceedings of the National Academy of Sciences of the United States of America*. 2015;112(39):12157-12162. doi:10.1073/PNAS.1516622112
93. Ma Y, Qiao X, Zeng R, et al. Irisin promotes proliferation but inhibits differentiation in osteoclast precursor cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2018;32(11):5813-5823. doi:10.1096/FJ.201700983RR
94. Juffer P, Jaspers RT, Klein-Nulend J, Bakker AD. Mechanically loaded myotubes affect osteoclast formation. *Calcified tissue international*. 2014;94(3):319-326. doi:10.1007/S00223-013-9813-8
95. Bakker AD, Jaspers RT. IL-6 and IGF-1 Signaling Within and Between Muscle and Bone: How Important is the mTOR Pathway for Bone Metabolism? *Current osteoporosis reports*. 2015;13(3):131-139. doi:10.1007/S11914-015-0264-1
96. Liu S, Gao F, Wen L, et al. Osteocalcin Induces Proliferation via Positive Activation of the PI3K/Akt, P38 MAPK Pathways and Promotes Differentiation Through Activation of the GPRC6A-ERK1/2 Pathway in C2C12 Myoblast Cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2017;43(3):1100-1112. doi:10.1159/000481752
97. Mera P, Laue K, Wei J, Berger JM, Karsenty G. Osteocalcin is necessary and sufficient to maintain muscle mass in older mice. *Molecular metabolism*. 2016;5(10):1042-1047. doi:10.1016/J.MOLMET.2016.07.002
98. Mera P, Laue K, Ferron M, et al. Osteocalcin Signaling in Myofibers Is Necessary and Sufficient for Optimum Adaptation to Exercise. *Cell metabolism*. 2016;23(6):1078-1092. doi:10.1016/J.CMET.2016.05.004
99. Magarò MS, Bertacchini J, Florio F, et al. Identification of Sclerostin as a Putative New Myokine Involved in the Muscle-to-Bone Crosstalk. *Biomedicines*. 2021;9(1):1-22. doi:10.3390/BIOMEDICINES9010071
100. Dumic J, Dabelic S, Flögel M. Galectin-3: an open-ended story. *Biochimica et biophysica acta*. 2006;1760(4):616-635. doi:10.1016/J.BBAGEN.2005.12.020
101. Menon RP, Hughes RC. Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. *European journal of biochemistry*. 1999;264(2):569-576. doi:10.1046/J.1432-1327.1999.00671.X
102. Seetharaman J, Kfanigsberg A, Slaaby R, Leffler H, Barondes SH, Rini JM. X-ray crystal structure of the human galectin-3 carbohydrate recognition domain at 2.1-A resolution. *The Journal of biological chemistry*. 1998;273(21):13047-13052. doi:10.1074/JBC.273.21.13047
103. Nakahara S, Oka N, Wang Y, Hogan V, Inohara H, Raz A. Characterization of the nuclear import pathways of galectin-3. *Cancer Research*. 2006;66(20):9995-10006. doi:10.1158/0008-5472.CAN-06-1772
104. Tsay YG, Lin NY, Voss PG, Patterson RJ, Wang JL. Export of galectin-3 from nuclei of digitonin-permeabilized mouse 3T3 fibroblasts. *Experimental cell research*. 1999;252(2):250-261. doi:10.1006/EXCR.1999.4643
105. Mehul B, Hughes RC. Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion. *Journal of cell science*. 1997;110 (Pt 10)(10):1169-1178. doi:10.1242/jcs.110.10.1169
106. Morris S, Ahmad N, Andre S, et al. Quaternary solution structures of galectins-1, -3, and -7. *Glycobiology*. 2004;14(3):293-300. doi:10.1093/GLYCOB/CWH029
107. Yang RY, Hill PN, Hsu DK, Liu FT. Role of the carboxyl-terminal lectin domain in self-association of galectin-3. *Biochemistry*. 1998;37(12):4086-4092. doi:10.1021/BI971409C

108. Ahmad N, Gabius HJ, André S, et al. Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *The Journal of biological chemistry*. 2004;279(12):10841-10847. doi:10.1074/JBC.M312834200
109. Brewer CF, Miceli MC, Baum LG. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. *Current opinion in structural biology*. 2002;12(5):616-623. doi:10.1016/S0959-440X(02)00364-0
110. Krześlak A, Lipińska A. Galectin-3 as a multifunctional protein. *Cellular & molecular biology letters*. 2004;9(2):305-328. Accessed January 22, 2022. https://pubmed.ncbi.nlm.nih.gov/15213811/
111. Matarrese P, Fusco O, Tinari N, et al. Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. *International journal of cancer*. 2000;85(4):545-554. doi:10.1002/(SICI)1097-0215(20000215)85:4<545::AID-IJC17>3.0.CO;2-N
112. Matarrese P, Tinari N, Semeraro ML, Natoli C, Iacobelli S, Malorni W. Galectin-3 overexpression protects from cell damage and death by influencing mitochondrial homeostasis. *FEBS letters*. 2000;473(3):311-315. doi:10.1016/S0014-5793(00)01547-7
113. Yang RY, Hsu DK, Liu FT. Expression of galectin-3 modulates T-cell growth and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(13):6737-6742. doi:10.1073/PNAS.93.13.6737
114. Elad-Sfadia G, Haklai R, Balan E, Kloog Y. Galectin-3 augments K-Ras activation and triggers a Ras signal that attenuates ERK but not phosphoinositide 3-kinase activity. *The Journal of biological chemistry*. 2004;279(33):34922-34930. doi:10.1074/JBC.M312697200
115. Oka N, Nakahara S, Takenaka Y, et al. Galectin-3 inhibits tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by activating Akt in human bladder carcinoma cells. *Cancer research*. 2005;65(17):7546-7553. doi:10.1158/0008-5472.CAN-05-1197
116. Lee YJ, Song YK, Song JJ, et al. Reconstitution of galectin-3 alters glutathione content and potentiates TRAIL-induced cytotoxicity by dephosphorylation of Akt. *Experimental cell research*. 2003;288(1):21-34. doi:10.1016/S0014-4827(03)00211-8
117. Galectin-3 â€“ A jack-of-all-trades in cancer | Elsevier Enhanced Reader. Accessed January 21, 2022. https://reader.elsevier.com/reader/sd/pii/S0304383511005337?token=1E221A54B58F4B96E1FCC60BC59A5FE7610E0738DD2845962B84331A6A5C85C99C2D32C3424F244C9FCD2B379C7424A6&originRegion=eu-west-1&originCreation=20220121224009
118. Park JW, Voss PG, Grabski S, Wang JL, Patterson RJ. Association of galectin-1 and galectin-3 with Gemin4 in complexes containing the SMN protein. *Nucleic acids research*. 2001;29(17):3595-3602. doi:10.1093/NAR/29.17.3595
119. Lin HM, Pestell RG, Raz A, Kim HRC. Galectin-3 enhances cyclin D(1) promoter activity through SP1 and a cAMP-responsive element in human breast epithelial cells. *Oncogene*. 2002;21(52):8001-8010. doi:10.1038/SJ.ONC.1205820
120. Kim HRC, Lin HM, Biliran H, Raz A. Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. *Cancer research*. 1999;59(16):4148-4154. Accessed January 23, 2022. https://pubmed.ncbi.nlm.nih.gov/10463621/
121. Shimura T, Takenaka Y, Fukumori T, et al. Implication of galectin-3 in Wnt signaling. *Cancer research*. 2005;65(9):3535-3537. doi:10.1158/0008-5472.CAN-05-0104
122. Shimura T, Takenaka Y, Tsutsumi S, Hogan V, Kikuchi A, Raz A. Galectin-3, a novel binding partner of beta-catenin. *Cancer research*. 2004;64(18):6363-6367. doi:10.1158/0008-5472.CAN-04-1816
123. Sève AP, Felin M, Doyennette-moyne MA, Sahraoui T, Aubery M, Hubert J. Evidence for a lactose-mediated association between two nuclear carbohydrate-binding proteins. *Glycobiology*. 1993;3(1):23-30. doi:10.1093/GLYCOB/3.1.23

124. van den Brule FA, Buicu C, Sobel ME, Liu FT, Castronovo V. Galectin-3, a laminin binding protein, fails to modulate adhesion of human melanoma cells to laminin. *Neoplasma*. 1995;42(5):215-219. Accessed January 23, 2022. https://pubmed.ncbi.nlm.nih.gov/8552198/
125. Sato S, Hughes RC. Binding specificity of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. *The Journal of biological chemistry*. 1992;267(10):6983-6990. doi:10.1016/s0021-9258(19)50525-7
126. Hikita C, Vijayakumar S, Takito J, Erdjument-Bromage H, Tempst P, Al-Awqati Q. Induction of terminal differentiation in epithelial cells requires polymerization of hensin by galectin 3. *The Journal of cell biology*. 2000;151(6):1235-1246. doi:10.1083/JCB.151.6.1235
127. Ochieng J, Warfield P, Green-Jarvis B, Fentie I. Galectin-3 regulates the adhesive interaction between breast carcinoma cells and elastin. *Journal of cellular biochemistry*. 1999;75(3):505-514. doi:10.1002/(sici)1097-4644(19991201)75:3<505::aid-jcb14>3.3.co;2-9
128. Probstmeier R, Montag D, Schachner M. Galectin-3, a beta-galactoside-binding animal lectin, binds to neural recognition molecules. *Journal of neurochemistry*. 1995;64(6):2465-2472. doi:10.1046/J.1471-4159.1995.64062465.X
129. Villa-Verde DMS, Silva-Monteiro E, Jasiulionis MG, et al. Galectin-3 modulates carbohydrate-dependent thymocyte interactions with the thymic microenvironment. *European journal of immunology*. 2002;32(5):1434-1444. doi:10.1002/1521-4141(200205)32:5<1434::aid-immu1434>3.0.co;2-m
130. Inohara H, Akahani S, Koths K, Raz A. Interactions between galectin-3 and Mac-2-binding protein mediate cell-cell adhesion. *Cancer research*. 1996;56(19):4530-4534. Accessed January 23, 2022. https://pubmed.ncbi.nlm.nih.gov/8813152/
131. Weilner S, Keider V, Winter M, et al. Vesicular Galectin-3 levels decrease with donor age and contribute to the reduced osteo-inductive potential of human plasma derived extracellular vesicles. *Aging*. 2016;8(1):16-33. doi:10.18632/AGING.100865
132. Simon D, Derer A, Andes FT, et al. Galectin-3 as a novel regulator of osteoblast-osteoclast interaction and bone homeostasis. *Bone*. 2017;105:35-41. doi:10.1016/J.BONE.2017.08.013
133. Bruder SP, Caplan AI. Cellular and molecular events during embryonic bone development. *Connective tissue research*. 1989;20(1-4):65-71. doi:10.3109/03008208909023875
134. Iacobini C, Fantauzzi CB, Pugliese G, Menini S. Role of Galectin-3 in Bone Cell Differentiation, Bone Pathophysiology and Vascular Osteogenesis. *International journal of molecular sciences*. 2017;18(11). doi:10.3390/IJMS18112481
135. Colnot C, Sidhu SS, Balmain N, Poirier F. Uncoupling of chondrocyte death and vascular invasion in mouse galectin 3 null mutant bones. *Developmental biology*. 2001;229(1):203-214. doi:10.1006/DBIO.2000.9933
136. Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. *Bone*. 1995;17(2 Suppl). doi:10.1016/8756-3282(95)00183-E
137. Aubin JE, Gupta AK, Bhargava U, Turksen K. Expression and regulation of galectin 3 in rat osteoblastic cells. *Journal of cellular physiology*. 1996;169(3):468-480. doi:10.1002/(sici)1097-4652(199612)169:3<468::aid-jcp7>3.0.co;2-m
138. Stock M, Schäfer H, Stricker S, Gross G, Mundlos S, Otto F. Expression of galectin-3 in skeletal tissues is controlled by Runx2. *The Journal of biological chemistry*. 2003;278(19):17360-17367. doi:10.1074/JBC.M207631200
139. Nakajima K, Kho DH, Yanagawa T, et al. Galectin-3 inhibits osteoblast differentiation through notch signaling. *Neoplasia (New York, NY)*. 2014;16(11):939-949. doi:10.1016/J.NEO.2014.09.005

➢ Figure references

Figure Number	Reference
1	Miller SC, de Saint-Georges L, Bowman BM, Jee WS. Bone lining cells: structure and function. Scanning Microsc. 1989 Sep;3(3):953-60; discussion 960-1. PMID: 2694361.
2	Sharon L. Truesdell, Marnie M. Saunders. Bone remodeling platforms: Understanding the need for multicellular lab-on-a-chip systems and predictive agent-based models[J]. Mathematical Biosciences and Engineering, 2020, 17(2): 1233-1252. doi: 10.3934/mbe.2020063
3	Neman J, Hambrecht A, Cadry C, Jandial R. Stem cell-mediated osteogenesis: therapeutic potential for bone tissue engineering. Biologics. 2012;6:47-57. doi: 10.2147/BTT.S22407. Epub 2012 Mar 9. PMID: 22500114; PMCID: PMC3324839.
4	Zheng CX, Sui BD, Qiu XY, Hu CH, Jin Y. Mitochondrial Regulation of Stem Cells in Bone Homeostasis. Trends Mol Med. 2020 Jan;26(1):89-104. doi: 10.1016/j.molmed.2019.04.008. Epub 2019 May 21. PMID: 31126872.
7	Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol. 2012 Apr 3;8(8):457-65. doi: 10.1038/nrendo.2012.49. PMID: 22473333.
8	Severinsen MCK, Pedersen BK. Muscle-Organ Crosstalk: The Emerging Roles of Myokines. Endocr Rev. 2020 Aug 1;41(4):594–609. doi: 10.1210/endrev/bnaa016. Erratum in: Endocr Rev. 2021 Jan 28;42(1):97-99. PMID: 32393961; PMCID: PMC7288608.
9	Kirk B, Zanker J, Duque G. Osteosarcopenia: epidemiology, diagnosis, and treatment-facts and numbers. J Cachexia Sarcopenia Muscle. 2020 Jun;11(3):609-618. doi: 10.1002/jcsm.12567. Epub 2020 Mar 22. PMID: 32202056; PMCID: PMC7296259.
10	• Protein data Bank (PDB) • Li YS, Li XT, Yu LG, Wang L, Shi ZY, Guo XL. Roles of galectin-3 in metabolic disorders and tumor cell metabolism. Int J Biol Macromol. 2020 Jan 1;142:463-473. doi: 10.1016/j.ijbiomac.2019.09.118. Epub 2019 Oct 8. PMID: 31604080.
11	Li YS, Li XT, Yu LG, Wang L, Shi ZY, Guo XL. Roles of galectin-3 in metabolic disorders and tumor cell metabolism. Int J Biol Macromol. 2020 Jan 1;142:463-473. doi: 10.1016/j.ijbiomac.2019.09.118. Epub 2019 Oct 8. PMID: 31604080.
12;13	Dumic J, Dabelic S, Flögel M. Galectin-3: an open-ended story. Biochimica et biophysica acta. 2006;1760(4):616-635. doi:10.1016/J.BBAGEN.2005.12.020