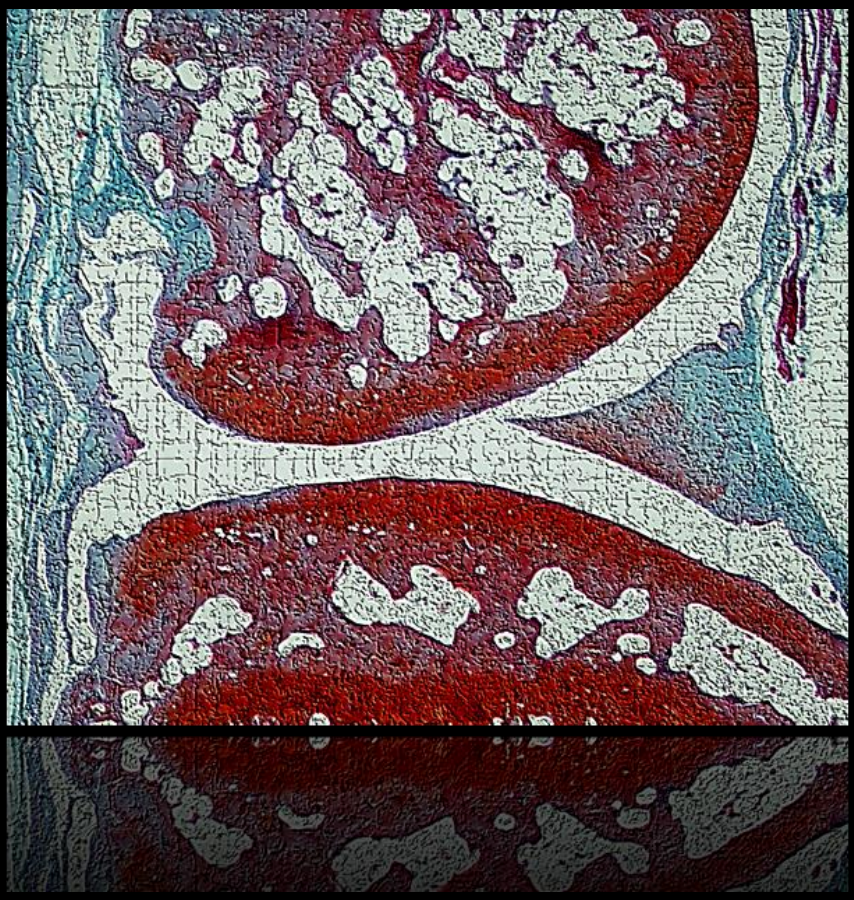

Maria Madalena Azevedo Alves Ribeiro

MECHANISMS INVOLVED IN DIABETES-ASSOCIATED OSTEOARTHRITIS: ROLE OF AUTOPHAGY

Tese de doutoramento em Ciências Farmacêuticas, ramo de Farmacologia e Farmacoterapia, orientada por
Professora Doutora Alexandrina Maria Ferreira dos Santos Pinto Mendes e por
Professor Doutor Francisco Javier Blanco García e apresentada à
Faculdade de Farmácia da Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

MECHANISMS INVOLVED IN DIABETES-ASSOCIATED OSTEOARTHRITIS: ROLE OF AUTOPHAGY

Maria Madalena Azevedo Alves Ribeiro

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra, para prestação de Provas de Doutoramento em Ciências Farmacêuticas, no ramo de Farmacologia e Farmacoterapia.

Faculdade de Farmácia

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Capa: Imagem de um corte de joelho de murganho db/db sujeito a cirurgia de indução de osteoartrite e tratamento com rapamicina durante 10 semanas. Coloração com safranina O. Ampliação 4x.

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Pretendo também manifestar publicamente o meu reconhecimento, pela ação atenta e estreito acompanhamento deste trabalho, à Doutora Beatriz Caramés Peréz.

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PUBLICATIONS

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Note: The results presented in this dissertation, included in Chapters 2 and 3, are formatted according to the style of the journal where the papers were published, with minor modifications.

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ABBREVIATIONS

4E-BP1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
AAOS	American Academy of Orthopaedic Surgeons
ACL	Anterior Cruciate Ligament
ACR	American College of Rheumatology
ADAMTS	A Disintegrin and Metalloproteinase with ThromboSpondin Motifs
AGEs	Advanced Glycation End Products
Akt/PKB	Protein Kinase B
AMPK	Adenosine 5 ´Monophosphate-Activated Protein Kinase
ANOVA	One-way Analysis of Variance
AP-1	Activator Protein-1
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments
Atgs	Autophagy-related proteins
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid Reagent Assay
Bcl-2	B Cell lymphoma 2
BH3	B Cell lymphoma 2 homology 3
BMI	Body Mass Index
BMPs	Bone Morphogenetic Proteins
C/EBPβ	CCAAT/Enhancer Binding Protein beta
CDMPs	Cartilage-Derived Morphogenetic Proteins
CI	Confidence Interval
CMA	Chaperone-Mediated Autophagy
COL10A1	Collagen Type X alpha 1
COMP	Cartilage Oligomeric Matrix Protein
COX-2	Cyclooxygenase-2
CTGF	Connective-Tissue Growth Factor

DAB	3,3-diaminobenzidine
DEPTOR	DEP Domain-Containing mTOR-Interacting Protein
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
E1	Ubiquitin Activating Enzyme
E2	Ubiquitin- Conjugating Enzyme
E3	Ubiquitin Ligase
ECM	Extracellular Matrix
eIF4E	Eukaryotic Translation Initiation Factor 4E
EMA	European Medicines Agency
ERK1/2	Extracellular Signal Regulated Protein Kinase 1/2
ESCEO	European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis
EULAR	European League Against Rheumatism
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factors
FIP200	Focal Adhesion kinase (FAK) Family Interacting Protein of 200kDa
FKBP12	FK506-binding protein 12
FLS	Fibroblast-Like Synoviocytes
FOXO	Forkhead-Box O
GAG	Glycosaminoglycan
GAP	GTPase-Activating Protein
GLUTs	Glucose Transporters
H&E	Hematoxylin and Eosin Staining
HA	Hyaluronic Acid
HC	Human Chondrocytes
ICMT	Intermittent Cyclic Mechanical Tension
IGF-1	Insulin-like Growth Factor-1

IL	Interleukin
IL-12	Interleukin-12
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
iNOS	Inducible form of Nitric Oxide Synthase
IRS	Insulin Receptor Substrate
IVD	Intervertebral Disc
JNK1	Jun-N-terminal Kinase 1
LC3	Microtubule-Associated Protein 1-Light Chain 3
LCL	Lateral Collateral Ligament
LIF	Leukemia Inhibitory Factor
LIR	LC3-interacting region
LKB1	Liver Protein Kinase B1
MAPK	Mitogen-Activated Protein Kinase
MCL	Medial Collateral Ligament
mLST8	Mammalian Lethal with sec-13 protein 8
MM	Medial Meniscus
MMPs	Matrix Metalloproteinases
MMTL	Medial Meniscotibial Ligament
MRI	Magnetic Resonance Imaging
mSin1	Mammalian Stress-Activated Map-Kinase Interacting Protein 1
mTOR	Mammalian Target of Rapamycin
NBR1	Neighbor of BRCA1 gene
NF-κB	Nuclear transcription Factor-kappaB
NGS	Normal Goat Serum
NO	Nitric Oxide
NP	Nucleus Pulposus

OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PDCD4	Programmed Cell Death 4
PE	Phosphatidylethanolamine
PGs	Proteoglycans
PGC-1α	Transcriptional Coactivator PPARγ Coactivator 1 alpha
PGE2	Prostaglandin-E2
PI3K	Phosphatidylinositol 3-Kinase
PIKK	Phosphatidylinositol Kinase-Related Kinase
PKC	Protein Kinase C
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PRAS40	Proline-Rich Akt Substrate 40kDa
Protor1/2	Protein Observed with Rictor 1 and 2
PtdIns	Phosphatidylinositol
PtdIns(3)P	Posphatidylinositol-3-Phosphate
Rag	Ras-related GTPase
RAGE	Receptor for Advanced Glycation End Products
Raptor	Regulatory-Associated Protein of Mammalian Target of Rapamycin
REDD	Regulated in Development and DNA Damage Responses 1
Rheb	Ras Homolog Enriched in Brain
Rictor	Rapamycin-Insensitive Companion of mTOR
ROS	Reactive Oxygen Species
RSK	p90 Ribosomal S6 kinase
RT	Room Temperature
S6K1	Ribosomal Protein S6 Kinase 1

SDS	Sodium Dodecyl Sulphate
SGK1	Serum/Glucocorticoid-induced Protein Kinase1
SIRT	Sirtuin
SNP	Sodium Nitroprusside
SYSADOA	Symptomatic Slow-Acting Drugs for Osteoarthritis
T2D	Type 2 Diabetes
TBST	Tris Buffered Saline Tween
TGF-β	Transforming Growth Factor-beta
TIMPs	Tissue Inhibitors of Metalloproteinases
TNF-α	Tumor Necrosis Factor alpha
TSC1-TSC2	Tuberous Sclerosis Heterodimeric Complex
ULK	Uncoordinated-51-like kinase
VEGF	Vascular Endothelial Growth Factor
Vps34	Vacuolar Protein Sorting Protein 34

ABSTRACT

Ongoing sociodemographic changes are leading to an increase in the prevalence of both Osteoarthritis (OA) and Type 2 Diabetes Mellitus (T2D), resulting in a tremendous socioeconomic burden. Since OA is a multifactorial disease with no efficient treatment options available, there is an urgent need to identify different OA phenotypes to establish effective therapies. Evidence from experimental and epidemiological data suggests an association between OA and diabetes: OA is more prevalent, more severe and has an earlier onset in diabetic patients. With this evidence a new OA phenotype emerges: diabetes-associated OA. Understanding the mechanisms underlying this association can be a key point in developing more effective therapies. Articular cartilage is a post-mitotic tissue with a low cell proliferation rate, depending on homeostatic mechanisms, especially autophagy, to maintain integrity and function. Autophagy is a catabolic process that is impaired in both OA and diabetes. However, the effect of diabetes on the regulation of autophagy in chondrocytes remains unknown.

In this sense, the aim of this thesis is to understand whether diabetic conditions facilitate OA progression and whether autophagy impairment plays a role in the process. We hypothesize that diabetes impairs articular cartilage integrity by directly affecting joint tissue cells and chondrocytes in particular, namely by decreasing autophagy. Another goal is to evaluate if pharmacological activation of autophagy has a protective effect and can constitute a therapeutic approach to prevent diabetes-accelerated OA. Although other forms of diabetes, namely type 1, may also affect joint tissues, and at least some mechanisms may be common, in this thesis we mainly focus in T2D.

To address these goals, we used an *in vitro* model mimicking hyperglycemia and hyperinsulinemia, the hallmarks of T2D, that also occur in type 1 diabetic patients under insulin therapy. Thus, human primary articular chondrocytes and cartilage explants and a chondrocytic cell line were cultured in a high glucose medium (25mM glucose) and exposed to high insulin concentrations (10 to 1000nM). Using this model, we found a decrease in autophagy markers that was partially mediated by increased activity of the Akt/mTOR pathway. The same diabetic conditions also led to proteoglycan loss, increased expression of the collagen-degrading enzyme, MMP-13, and of the pro-inflammatory cytokine, IL-1β, suggesting a pro-catabolic effect in chondrocytes and articular cartilage. Moreover, pharmacological autophagy activation, by inhibiting mTOR with Rapamycin, was also found to be effective in preventing the decrease in autophagy markers and the increase in inflammation observed under diabetic conditions. Importantly, we also found decreased basal levels of autophagy markers, namely LC3, and increased activity of the mTOR pathway as revealed by the levels of its downstream target, phospho-rpS6, in chondrocytes from diabetic OA

patients compared to non-diabetic OA patients. Taken together, these findings support our hypothesis that a reduction of autophagy is an important mechanism by which diabetes favors cartilage degradation.

To further corroborate these results and evaluate their relevance under more complex dynamic conditions closer to the human disease, we performed an *in vivo* study by surgically inducing OA in obese diabetic mice (db/db mice) and in non-diabetic lean mice. All animals were subjected to surgical OA induction by transection of the medial meniscotibial and the medial collateral ligaments in the right knee. The left knee was employed as a control. Mice were divided into three groups: untreated Lean, Vehicle-treated db/db, and Rapamycin-treated db/db mice. Rapamycin (2mg/kg weight) or Vehicle (dimethyl sulphoxide) were administered intraperitoneally, 3 times a week, for 10 weeks. After OA induction, Vehicle-treated db/db mice elicited increased cartilage damage and synovial inflammation, up-regulation of OA biomarkers (MMP-13), as well as decreased cartilage cellularity, compared to lean mice. Moreover, LC3 staining was also reduced in db/db mice. These results suggest that T2D compromises autophagy and accelerates experimental OA. Importantly, autophagy activation by Rapamycin treatment improved metabolic parameters, attenuated cartilage degradation and synovial inflammation, decreased catabolic (MMP-13) and inflammatory (IL-12) markers, and increased cartilage cellularity, in db/db mice with OA. Rapamycin also increased LC3 staining in joint tissues. No histopathological differences in the left control knee were observed among the three groups. These results show that autophagy activation reduces the severity of experimental OA in db/db mice.

In conclusion, the results obtained show that diabetic conditions impair chondrocyte autophagy, both *in vitro* and *in vivo*, and this facilitates OA development and progression, suggesting that impaired autophagy can be one of the mechanisms involved in diabetes-associated OA. Moreover, pharmacological activation of autophagy through mTOR inhibition is sufficient to protect against diabetes-induced joint destruction. Therefore, pharmacological autophagy activation deserves to be further studied as a potential new strategy to prevent or stop OA progression in diabetic patients.

RESUMO

Devido às alterações no perfil sociodemográfico da população, a Osteoartrite (OA) e a Diabetes Mellitus tipo 2 (DM tipo 2), são agora altamente prevalentes, com um enorme impacto socioeconómico. A OA é uma doença multifatorial para a qual se desconhecem terapias eficazes, sendo por isso urgente identificar fenótipos específicos da OA que permitam desenvolver terapêuticas mais eficientes. Estudos epidemiológicos e experimentais sugerem uma associação entre OA e diabetes: OA é mais prevalente, mais severa e com início mais precoce em doentes diabéticos do que em não diabéticos. Esta evidência suporta a existência de um fenótipo específico da OA induzido pela diabetes. Perceber os mecanismos subjacentes a esta associação pode ser essencial para desenvolver terapias mais específicas. A cartilagem articular é um tecido pós-mitótico com uma baixa taxa de proliferação celular, dependendo de mecanismos homeostáticos, nomeadamente da autofagia, para manter a sua integridade e função. A autofagia é um processo catabólico que se encontra alterado tanto na OA como na diabetes. Contudo, os efeitos da diabetes na regulação da autofagia dos condrócitos permanecem por esclarecer.

Neste contexto, o objetivo dessa tese é perceber se as condições diabéticas facilitam a progressão da OA e se o comprometimento da autofagia tem um papel importante neste processo. A nossa hipótese é de que a diabetes compromete a integridade da cartilagem através do seu efeito nas células dos tecidos articulares, em particular nos condrócitos, nomeadamente por redução na autofagia. Outro objetivo é avaliar se a ativação farmacológica da autofagia tem um efeito protetor e se pode constituir uma abordagem terapêutica para prevenir a OA associada à diabetes. Apesar de outras formas de diabetes, nomeadamente a DM tipo 1, poderem também afectar tecidos articulares, e pelo menos alguns dos mecanismos responsáveis poderem ser comuns, esta tese foca-se na DM tipo 2.

Para atingir estes objetivos, foi utilizado um modelo *in vitro* simulando a hiperglicemia e hiperinsulinemia, características típicas da DM tipo 2 que também podem ser encontradas em doentes DM tipo 1 insulino-tratados. Assim, condrócitos humanos e explantes de cartilagem e uma linha de condrócitos foram cultivados num meio com alta glucose (25mM) e expostos a altas concentrações de insulina (10-1000nM). Usando este modelo, uma diminuição de marcadores de autofagia que, pelo menos em parte, foi mediada pelo aumento de atividade da via Akt/mTOR, foi observada. Estas condições diabéticas conduziram ainda a uma perda de proteoglicanos, a um aumento da expressão da enzima de degradação, MMP-13, e da citoquina pró-inflamatória, IL-1β, sugerindo um efeito pro-catabólico nos condrócitos e na cartilagem. A ativação farmacológica da autofagia, através da inibição do mTOR com a rapamicina, demonstrou prevenir a diminuição dos

marcadores da autofagia e o incremento da inflamação, observados nestas condições diabéticas. Para além disso, foi observada uma diminuição nos níveis basais de marcadores da autofagia,LC3, e um aumento da actividade da via mTOR, detectado pelos níveis de p-rpS6, nos condrócitos de doentes OA diabéticos comparados com condrócitos de doentes OA não-diabéticos. No seu conjunto, estes resultados suportam a nossa hipótese de que uma diminuição da autofagia é um mecanismo importante pela qual a diabetes favorece a degradação da cartilagem.

Para confirmar estes resultados e avaliar a sua relevância num contexto mais próximo do encontrado nos humanos, um estudo *in vivo* foi realizado. Assim, a OA foi induzida cirurgicamente, pela seção dos ligamentos meniscotibial e colateral medial, no joelho direito de murganhos obesos diabéticos (db/db), e murganhos lean não diabéticos. O joelho esquerdo foi utilizado como controlo. Os murganhos foram divididos em três grupos: lean não tratados, db/db tratados com veículo, e db/db tratados com rapamicina. A rapamicina (2mg/kg peso) ou o veículo (dimetilsulfóxido) foram administrados por via intraperitoneal, 3 vezes por semana, durante 10 semanas. Após a indução da OA, os murganhos db/db tratados com veículo apresentaram um aumento na degradação da cartilagem, na inflamação sinovial, e na expressão biomarcadores da OA (MMP-13), e uma diminuição da celularidade, quando comparados com os murganhos lean. Uma diminuição na expressão de LC3 foi também observada nos murganhos db/db. Estes resultados mostram que a DM tipo 2 compromete a autofagia e acelera a OA induzida experimentalmente. Por sua vez, a ativação da autofagia induzida pela administração de rapamicina melhorou os parâmetros metabólicos, diminuiu a degradação da cartilagem e a inflamação sinovial, diminuiu marcadores catabólicos (MMP-13) e inflamatórios (IL-12), e aumentou a celularidade da cartilagem. A rapamicina aumentou também a expressão de LC3 nos tecidos articulares. Em nenhum dos grupos foram detetadas diferenças histológicas no joelho esquerdo (controlo). Estes resultados demonstram que a ativação farmacológica da autofagia atenua a severidade da OA induzida experimentalmente em murganhos db/db.

Em suma, os resultados obtidos mostram que as condições diabéticas alteram a autofagia dos condrócitos, tanto *in vitro* como *in vivo*, e que isso facilita o desenvolvimento e progressão da OA, apontando a alteração da autofagia como um dos possíveis mecanismos envolvidos na degradação da cartilagem induzida pela diabetes. Este estudo demonstra ainda que a ativação farmacológica da autofagia, pela inibição do mTOR, é suficiente para proteger contra a degradação da articulação induzida pela diabetes. Assim, a ativação farmacológica da autofagia surge como uma potencial estratégia para prevenir ou parar a progressão da OA em doentes diabéticos.

CHAPTER 1

INTRODUCTION

1.1 ARTHRITIS

Arthritis is a general term for many diseases meaning joint inflammation (arthro= joint, itis=inflammation), and it can affect any synovial joint. There are more than 100 different types of arthritis, with different causes, severity, location of symptoms and treatment options, such as Osteoarthritis, Rheumatoid Arthritis, Psoriatic Arthritis, Gout, Lupus, and Carpal Tunnel Syndrome. Osteoarthritis is the most common type of arthritis. Typically, symptoms of arthritis develop over time and include pain, stiffness, and decreased range of motion.

1.2 SYNOVIAL JOINT

Joint is a specialized structure, a point of union of two or more bones, which allows stability and movement. There are different types of joints, synovial joints being the most frequent in the body. Synovial joints make pain-free movement possible and are composed of multiple structures: an articular capsule, continuous with periosteum that surrounds joint cavity; a synovial membrane - thin membrane that covers the joint cavity, responsible for the secretion of synovial fluid to maintain joint lubrication and provide nutrition to the articular cartilage; and hyaline articular cartilage which covers the end of bones in a joint. Other tissues such ligaments, muscles and tendons may be also present and provide additional support to synovial joints (Buckwalter et al, 2005; Khan et al, 2007). The structures of the synovial joint are presented in figure 1.1.

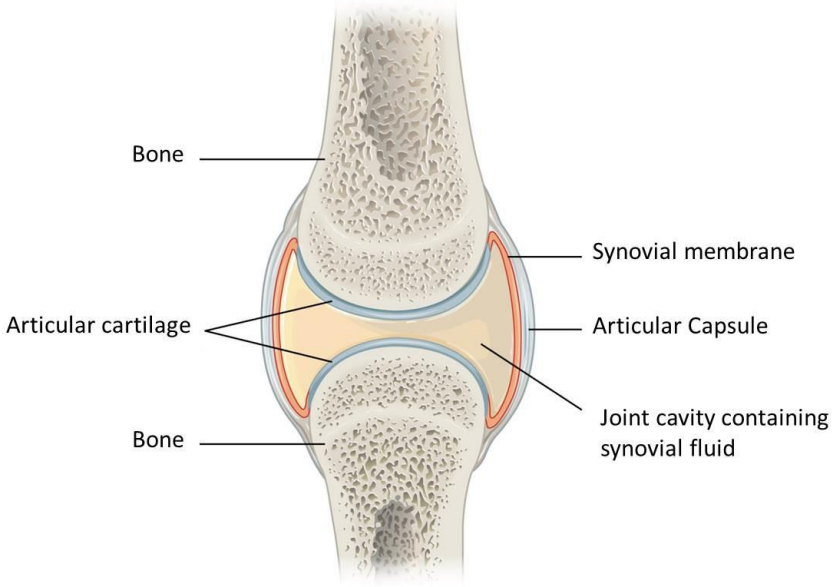

Figure 1. 1- **Schematic representation of the major structures of a normal synovial joint.** (Available online http://cnx.org/contents/6c5b58ca-6c6d-4ab3-8b41-60c8733a3144@4)

1.2.1 ARTICULAR CARTILAGE

Articular cartilage is a specialized, avascular, aneural and alymphatic connective tissue (Martel-Pelletier et al, 2008). Despite being only a few millimeters thick -2 to 4mm- it provides a coating material that offers low friction surface in joint motion by absorbing and facilitating the transmission of mechanical load, which minimizes the stress on the subchondral bone, protecting the bone from abrasion. (Buckwalter et al, 2005; Martel-Pelletier et al, 2008; Sophia Fox et al, 2009). Its functions and properties are related to the composition and structure of the extracellular matrix (ECM). Thus, maintaining cartilage integrity is essential for the normal function of the articular cartilage.

1.2.1.1 COMPONENTS OF THE ARTICULAR CARTILAGE

Articular cartilage is composed by a unique cell type – chondrocytes - immersed in an abundant ECM.

A) Chondrocytes

Chondrocytes represent only 2-5% of the total tissue volume (Goldring, 2006). They are responsible for the synthesis, maintenance and degradation of the ECM. According to the anatomical

region of articular cartilage where they are located, they have different shape, size, and metabolic activity with a different pattern of gene expression (Buckwalter et al, 2005; Goldring & Marcu, 2009). Chondrocytes are surrounded by the pericellular matrix, a thin layer that contains non-fibrillar material such as proteoglycans (PGs). Surrounding the pericellular matrix there is a territorial matrix, formed by a dense network of collagen fibers, especially type VI, that form a "capsule-like structure" around the chondrocytes, which protects the chondrocytes from mechanical stress. Finally, there is the interterritorial region, the largest one, composed by a fibrillar collagen network associated with aggrecan and other small PGs, which contributes for the biomechanical properties of cartilage: the resilience (Goldring & Goldring, 2010; Martel-Pelletier et al, 2008; Sophia Fox et al, 2009). Chondrocytes live in an avascular environment, with low levels of nutrients and oxygen. As a consequence, chondrocyte metabolism operates at low oxygen tension, ranging from 10% at the surface to 1% in deep zone, mainly supplied by the synovial fluid due to a lack of blood vessels (Goldring, 2006). Low oxygen tension, the more physiological environment for chondrocytes, is the best environment, where they can synthetize higher amounts of PGs and collagen, and reduce the mRNA expression and protein release of catabolic enzymes (Strobel et al, 2010). In order to adapt to these low oxygen concentrations, healthy chondrocytes obtain energy from glucose, mainly through the glycolytic pathway (Lee & Urban, 1997). Despite being normally quiescent, they are able to respond to different biochemical, physical or structural stimuli by synthetizing enzymes, growth factors, cytokines and the components of ECM, and alter negative or positively articular homeostasis (Goldring, 2006). However, the low number and the low rate of proliferation of chondrocytes, which adding to the tissue´s lack of nerves and blood vessels and the low turnover of the matrix components, means that cartilage has an impaired ability to self-repair (Fosang & Beier, 2011).

B) Extracellular Matrix

Extracellular Matrix (ECM) represents 95% of the dry weight of articular cartilage. ECM is a specialized matrix of collagens, PGs, and water. Noncollagenous proteins and glycoproteins are also present but in lesser amounts. ECM plays a crucial role in regulating chondrocyte function and in the maintenance of the osmotic environment of chondrocytes. The organized ECM is essential for tissue function (Alford & Cole, 2005).

The water content in articular cartilage varies through the depth of the tissue. The highest concentration, around 80%, is found near the cartilage surface, decreasing to around 65% in the deep zone. The diffusion of water through the cartilage helps nutrients to move from the synovial fluid through the tissue, contributing to the nutrition and lubrication of the tissue, and helps to maintain its resilience (Martel-Pelletier et al, 2008).

Collagen is a fibrous protein that represents the most abundant macromolecule in the ECM (60 to 70% of the dry weight). Type II collagen is the primary collagen in this tissue, representing 90-95% of the collagen in the ECM, but other types of collagen such as VI, IX, and XI are also present. Type IX and XI collagens stabilize the collagen fibrils assembled from type II collagen, while type VI collagen, mainly present in territorial matrix, anchor chondrocytes to the matrix (Buckwalter et al, 2005). Further collagen types, including types III, XII and XIV, are also found but in smaller amounts (Eyre, 2002). Type X collagen only appears in the calcified cartilage zone of articular cartilage or in the hypertrophic zone of the growth plate (Camarero-Espinosa et al, 2016). Collagens are composed of a triple helix of three identical polypeptide chains (α- chains) which provide the shear and tensile strength to maintain the integrity of articular cartilage. Overall, the collagen forms a highly organized fibrillary network that immobilizes proteoglycan aggregates and serves as a tensile element (Martel-Pelletier et al, 2008).

PGs are macromolecules synthetized by chondrocytes and constitute the second largest group of the ECM components, accounting to 10- 15% of the tissue wet weight (Sophia Fox et al, 2009). They consist of glycosaminoglycans (GAG) – long unbranched polysaccharide made of repeating disaccharide units – covalently attached to a central core protein. The glycosaminoglycan chains are composed of negatively charged carboxyl or sulfated groups, such as hyaluronic acid (HA), keratin sulfate, and chondroitin sulfate, which attract positively charged ions. This leads to different ion concentrations between articular cartilage and surrounding tissues. As a consequence of this osmotic imbalance, water is drawn into the tissue causing swelling and expansion of matrix network, crucial for the biomechanical properties of cartilage (Buckwalter et al, 2005; Kiani et al, 2002). A variety of PGs is present in articular cartilage, aggrecan being the largest and most abundant (90% of the total cartilage matrix proteoglycan mass). Aggrecan, that fills most of the interfibrillar space of the matrix cartilage, possesses more than one hundred chondroitin sulfate and keratan sulfate chains that are associated with HA to form multimeric aggregates via link protein, a protein that stabilizes this interaction. Aggrecan provides an extremely high fixed charge density, creating a high osmotic environment necessary to retain the water needed for nutrient and solute transport (Dudhia, 2005; Heinegard, 2009). Noncollagenous proteins and glycoproteins, such as Biglycan, Decorin, Fibromodulin, Matrilins, and Cartilage Oligomeric Matrix Protein (COMP) are also present in articular cartilage, but in small amounts (Roughley, 2001). Although these proteins are less studied, they have a role in tissue assembly and in maintaining the properties and functions of the tissue (Buckwalter et al, 2005; Heinegard, 2009). Structure and composition of ECM of articular cartilage are presented in figure 1.2.

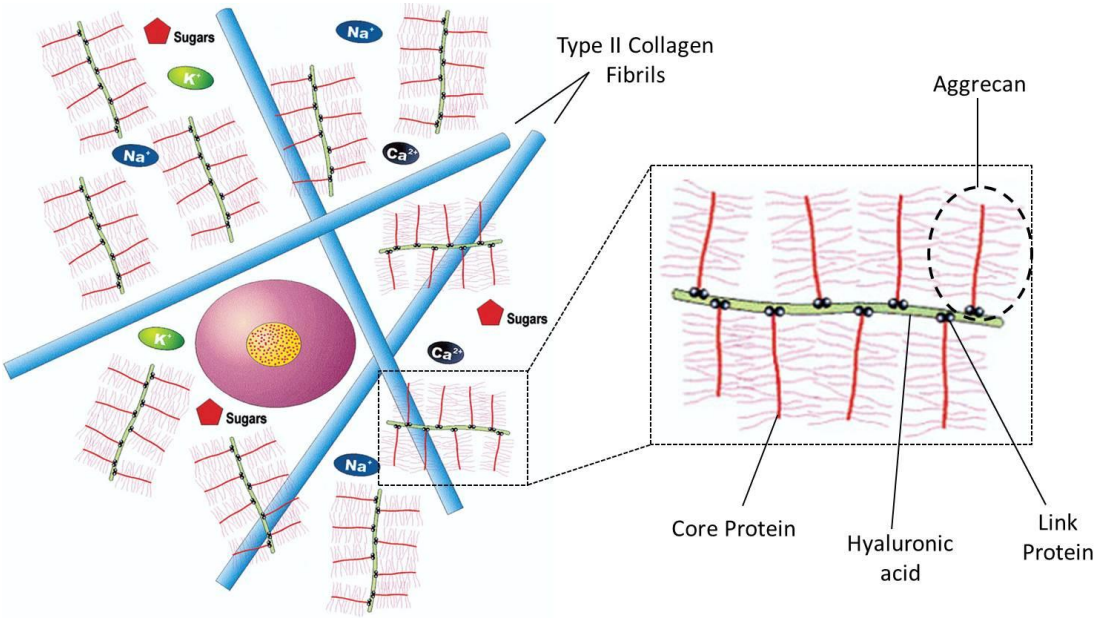

Figure 1. 2 **- Schematic representation of an articular chondrocyte surrounded by ECM in a healthy articular cartilage.** Adapted from (Mobasheri et al, 2002).

The composition and structure of articular cartilage is heterogeneous, with differences in cell morphology, density, and organization of its components. Thus, articular cartilage can be divided into four zones- superficial, middle, deep and calcified zone- each one with different properties and functions, presented in figure 1.3.

- The superficial zone is the thinnest one, which represents 10 to 20% of articular thickness. It is composed of collagen fibrils oriented in parallel to the articular surface, low proteoglycan content, and a high number of elongated chondrocytes, also aligned in parallel to the surface. This zone contacts with synovial fluid and is responsible for protecting and maintaining the deeper layers, providing resistance to shear, tensile and compressive forces established by movement (Martel-Pelletier et al, 2008; Sophia Fox et al, 2009).

- The middle zone, next to the superficial zone, represents 40 to 60% of the total cartilage volume. It contains PGs, thick collagen fibrils oriented obliquely and chondrocytes. Chondrocytes in this zone are more spherical and less abundant. This zone provides resistance to compressive forces (Blalock et al, 2015; Sophia Fox et al, 2009).

- The deep zone represents 30% of the total cartilage volume. It contains the largest collagen fibrils - aligned perpendicular to articular surface - the highest PGs content, and the lowest water concentration, making this layer responsible of resisting compressive forces. The chondrocytes are rounded, arranged in columnar orientation, and perpendicular to articular surface (Blalock et al, 2015; Sophia Fox et al, 2009).

- The calcified cartilage zone is separated from the deep zone by the tidemark. This calcified layer separates articular cartilage from the subchondral bone and is responsible of ensuring firm anchorage between the collagen fibrils of articular cartilage and subchondral bone. This layer presents rounded, scarce and hypertrophic chondrocytes, without PGs (Blalock et al, 2015; Martel-Pelletier et al, 2008; Poole, 1997; Sophia Fox et al, 2009).

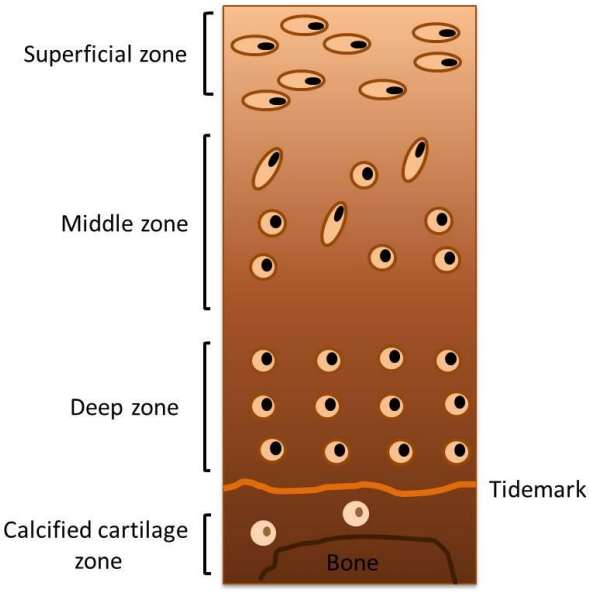

Figure 1. 3 - **Schematic representation of the different zones of a normal articular cartilage and chondrocyte distribution.** Adapted from (Grogan et al, 2009).

Maintaining this normal structure of articular cartilage is crucial for functional cartilage. On the other hand, when proteolysis of matrix components occurs, the swelling pressure of aggrecan is disturbed and as a consequence, the capacity of cartilage to support and redistribute compressive forces is compromised (Fosang & Beier, 2011).

1.2.1.2 DEGRADATION OF ARTICULAR CARTILAGE

Under normal conditions, chondrocytes have the capacity to maintain the equilibrium between synthesis and degradation of ECM components, thus maintaining the remodeling of articular cartilage. As articular cartilage is an avascular tissue, normal mechanical load plays an important role in cartilage homeostasis because it leads to fluid movement between cartilage and synovial fluid, which permits the diffusion of molecules and facilitates nutrition and elimination of waste metabolic products (Maroudas et al, 1968; O'Hara et al, 1990). However, as a consequence of various factors, such as aging, joint disease or abnormal mechanical load, this balance can be disrupted, and the catabolic process exceed the anabolic process. This leads to an increased loss of PGs, collagens and to a decrease in the deposition of new molecules. As a result of the failure of this

balance, progressive cartilage degradation leads to impairment of cartilage structure and function (Lee et al, 2013; Loeser, 2008). Importantly, one of the most common diseases displaying this type of imbalance is Osteoarthritis.

1.3 OSTEOARTHRITIS

Osteoarthritis (OA) is the most common form of arthritis (Allen & Golightly, 2015; Neogi & Zhang, 2013). It is considered a heterogeneous disease that affects millions of people worldwide; with 10-15% of adults over 60 years suffering from some degree of OA (Haq et al, 2003; Heidari, 2011; Johnson & Hunter, 2014). According to the World Health Organization it is considered a priority disease, being the fifth highest cause of years lost to disability in developed countries and the ninth highest cause in developing countries (Kaplan et al, 2013). Although OA can occur in any joint, the knee, hip, hand, spine and foot are the most commonly affected. OA causes incapacity and disability in adults; and reduces quality of life and the patients´ participation in social activity, representing an enormous socioeconomic impact. Accordingly, around 80% of those with OA have some limitation in movement and 25% cannot perform major activities of daily life (Hunter et al, 2014).

Altman R.D. and the Diagnostic and Therapeutic Subcommittee of the American Rheumatism Association published in 1987 a “Criteria for the Classification of Osteoarthritis of the knee and Hip” where defined OA as “heterogenous group of conditions leading to joint symptoms and signs associated with defective integrity of articular cartilage in addition to associated changes in the underlying bone and at the joint margins” (Altman, 1987). Since then a variety of definitions emerged but there is a consensus that OA is a heterogeneous disease considered as a whole joint disease. Nowadays, Osteoarthritis Research Society International (OARSI) defines OA as “a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness” (Kraus et al, 2015). In fact, the first events observed in OA occur in cartilage, at the joint surfaces areas, but structures around articular cartilage are also affected, including synovium, ligaments, muscles, meniscus, intra-articular fat, and subchondral bone (Felson et al, 2000; Malfait, 2016). Thus, phenotypic changes in cells of superficial layer, fibrillation with progressive loss of articular cartilage, cartilage calcification, subchondral bone remodeling, sclerosis, osteophyte formation, and mild to moderate inflammation in synovial lining are some

characteristic features present in OA (Bijlsma et al, 2011; Goldring & Berenbaum, 2015). Some of these characteristic features are presented in figure 1.4.

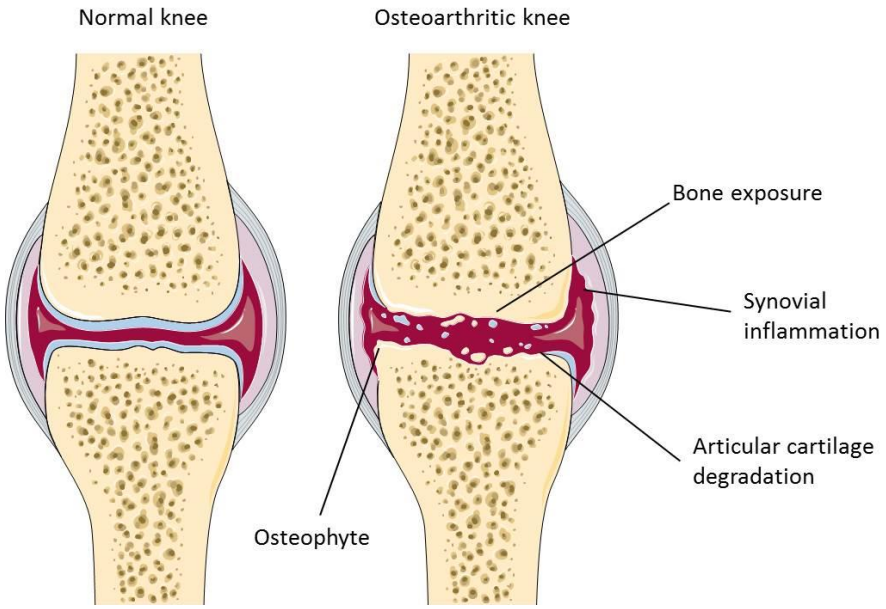

Figure 1. 4 - **Schematic representation of a normal and an osteoarthritic knee.** (Adapted from http://www.servier.com)

1.3.1 PATHOPHYSIOLOGICAL MECHANISMS OF OSTEOARTHRITIS

As previously stated, under normal conditions, chondrocytes are able to respond to different cues in order to maintain the homeostatic balance between anabolic and catabolic factors. However, when this equilibrium becomes unbalanced, failure of cartilage homeostasis occurs and ultimately leads to OA.

In the early stages of OA, articular cartilage changes occur at the joint surface. In these stages, chondrocytes exhibit a temporary proliferative response with cell proliferation resulting in cluster formation, and increased synthetic activity with induction of ECM synthesis, in an attempt to regenerate the damaged ECM and maintain cartilage integrity. However, catabolic factors such as matrix-degrading enzymes are also increased (Favero et al, 2015; Goldring, 2012; Goldring & Goldring, 2010; Goldring et al, 2011). At some point the anabolic activity is unable to compete with catabolic activity and the regeneration process fails, resulting in further amplification of the catabolic activity and damage progression. As the process continues, the increased catabolic activity, mediated by an up-regulation of cartilage-degrading proteinases and inflammatory mediators, occurs in conjunction with a decrease in matrix metalloproteinase enzyme inhibitors and ECM synthesis. This leads to a gradual loss of PGs followed by type II collagen degradation and a progressive cartilage

destruction (Sandell & Aigner, 2001). Although the loss of PGs can be reversible, the degradation of the collagen network cannot be regenerated, making this a critical point for cartilage integrity disruption (Goldring & Berenbaum, 2015). These changes are accompanied by a shift in chondrocyte phenotype towards a hypertrophy-like state followed by cartilage calcification (Lories & Luyten, 2011). As a consequence of this altered composition and structure, the cartilage layer becomes thinner and the compressive resistance and osmotic pressure within the tissue are compromised (Maroudas, 1976). Eventually the matrix becomes unable to resist to stress, leading to fibrillation of the superficial zone, surface erosion, and appearance of deeper fissures that ultimately result in full depth cartilage destruction (Favero et al, 2015; Xia et al, 2014). Beyond this cartilage destruction, other histological changes are characteristic, including tidemark duplication with vascular penetration of the calcified cartilage from subchondral bone, increase in subchondral bone remodeling and osteophyte formation (Goldring & Goldring, 2010; Houard et al, 2013; Lories & Luyten, 2011). Moreover, although it is not always present, synovial inflammation can be also observed in OA (Berenbaum, 2013; Goldring & Otero, 2011). The inflammatory mediators produced in the synovium can disperse through synovial fluid and reach the cartilage and activate chondrocytes, while the reverse is also possible, for instance, when chondrocytes suffer trauma or mechanical stress. The increase in the synthesis of inflammatory mediators in cartilage and in synovium, will amplify the inflammatory response and create a positive feedback cycle that sustains the synovitis, contributing to an exacerbation of cartilage degradation (Scanzello & Goldring, 2012). In fact, synovial inflammation plays a critical role in the clinical presentation, namely contributing to swelling, pain and joint stiffness (Siebuhr et al, 2016).

Different catabolic and anabolic factors are involved in OA pathogenesis. Catabolic factors include proteases, fibronectin, neuromediators, and inflammatory mediators (Martel-Pelletier et al, 2008). Proteases are responsible for the proteolytic cleavage of the articular cartilage components, and are synthetized by chondrocytes upon various stimuli (Mort & Billington, 2001). Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with trombospondin motifs (ADAMTS) are the most common proteases involved in OA, responsible for the breakdown of collagen and aggrecan, respectively (Blanco Garcia, 1999; Mort & Billington, 2001). Several members of the ADAMTS family (ADAMTS-1,-4,-5,-8,-9 and -15) are present in articular cartilage, with ADAMTS-4 and ADAMTS-5 being the most important in OA (Burrage et al, 2006; Song et al, 2007). Likewise, there are several members of MMPs family and they can be divided in different groups according to substrate selectively and tissue location: collagenases (MMP-1, -8, and -13) that are active against native fibrillar collagens (types I, II and III); gelatinases (MMP-2, and -9) with high affinity to denatured collagen and types IV and V collagen; stromelysins able to degrade non-collagen

components of ECM (MMP-3, -10, and -11), and membrane type MMPs (MT-MMP) (MMP-14, -15, -16, -17, -24, and -25) (Burrage et al, 2006). Despite all collagenases can cleave the triple helix, the most important MMP involved in OA seems to be MMP-13, which preferentially cleaves type II collagen (Billinghurst et al, 1997; Takaishi et al, 2008). Although OA is not considered a classical inflammatory disease, the expression of inflammatory mediators has been well documented in OA cartilage and it is well recognized that inflammation contributes to OA. The mechanisms responsible for initiating inflammation in OA remain unclear, however, it appears that mechanical and metabolic factors may be involved. Inflammatory mediators can be produced by any joint tissue and can reach other tissues through synovial fluid, activating a low grade chronic inflammatory response that drives and perpetuates joint destruction. Indeed, inflammatory mediators act on chondrocytes and cause further catabolism by increasing the production of proteases and others cytokines and inflammatory factors, and by decreasing the synthesis of ECM components, such as type II collagen and aggrecan, and the synthesis of tissue inhibitors of metalloproteinases (TIMP) (Lopez-Armada et al, 2007; Sandell & Aigner, 2001). Among other pro-inflammatory cytokines, Interleukin-1β (IL-1β) and Tumor Necrosis Factor- α (TNF-α) take the lead role in OA (Blanco Garcia, 1999; Goldring & Goldring, 2004; Moos et al, 1999). In fact, OA patients exhibit increased levels of both cytokines in synovial fluid, synovial membrane, subchondral bone and cartilage (Kapoor et al, 2011). The catabolic effects of these cytokines are multiple and include the synthesis and release of the MMPs, eicosanoids (prostaglandins -PGE2- and leukotrienes), nitric oxide (NO), and also induce their own production and that of other pro-inflammatory cytokines (such as IL-6, Leukemia Inhibitory Factor (LIF), IL-17, and IL-18) and chemokines, all contributing to an increase in cartilage degradation. Many of these factors will function independently or operate in a synergistic manner to promote catabolic reactions, thus contributing to OA initiation and progression (Goldring, 2000; Goldring & Goldring, 2007; Rahmati et al, 2016).

On the other hand, anabolic factors are responsible for stimulating ECM matrix synthesis. The most relevant in articular cartilage include insulin-like growth factor-1 (IGF-1), Bone Morphogenetic Proteins (BMPs), Cartilage-Derived Morphogenetic Proteins (CDMPs), Transforming Growth Factor β1 (TGF-β1), Connective-Tissue Growth Factor (CTGF) and Fibroblast Growth Factor (FGF)(Goldring, 2000; Martel-Pelletier et al, 2008).

1.3.2 RISK FACTORS AND OSTEOARTHRITIS PHENOTYPES

During many years OA was considered as a disease related only to aging and to the mechanical stress of joints, without considering other risk factors. Recently this concept changed and OA is now considered a disease where many interrelated mediators such as metabolic, hormonal and

humoral, can contribute to the initiation and progression of the disease (Velasquez & Katz, 2010). Currently, OA is considered a multifactorial disease, with different prognosis and clinical courses, whose causes are not yet completely understood. The development and progression of the disease can be a result of the interaction between various systemic and local factors that include age, sex, ethnicity and race, genetics, nutrition, osteoporosis, smoking, obesity and metabolic disease, sarcopenia, and local mechanical risk factors (Musumeci et al, 2015a). Thus, based on different risk factors, OA can now be differentiated and classified according to a variety of phenotypes including metabolic, age-related, inflammatory, hormonal, pain-related, genetic and injury-related phenotypes (Blanco, 2014; Zhuo et al, 2012). Many efforts have been made to understand the mechanisms involved in the initiation and progression of OA in different phenotypes. Although different phenotypes can be associated with different clinical manifestations they all lead to an OA syndrome with joint destruction. To this moment there are no efficient treatments to stop OA progression. This failure may be a consequence of viewing OA without considering the different OA phenotypes (Castaneda et al, 2014; Felson, 2010). Therefore, identifying the risk factors that result in distinct OA phenotypes is essential to divide patients into different subgroups. It is likely that these different phenotypes may need different therapeutic approaches. In fact, understanding the underlying mechanisms of disease progression can help differentiate OA phenotypes, and thus provide the right treatment to the right patient. This knowledge could enable the development of more effective, tailored therapies (Felson, 2010; Lane et al, 2011; Malfait, 2016).

Although ageing remains one of the most important risk factors for OA, metabolic phenotype has become the second most frequent subtype of OA, and so, nowadays, it is the one that raises more interest among the scientific community (Zhuo et al, 2012). No universally accepted definition of metabolic syndrome exists but there is a general consensus regarding its main components, which include insulin resistance, hyperglycemia or impaired glucose tolerance, visceral obesity, dyslipidemia, and hypertension (Bonomini et al, 2015; Guo, 2014). In fact, a higher prevalence of metabolic syndrome in OA patients was reported (Puenpatom & Victor, 2009). There is also published evidence describing a link between the rate of OA initiation and progression, OA pain, and need of arthroplasty and the accumulation of metabolic components (Jungmann et al, 2013; Monira Hussain et al, 2014; Shin, 2014; Sowers et al, 2009; Yoshimura et al, 2011; Yoshimura et al, 2012). However, the validity of this association should be interpreted with caution since many studies reported on the findings of a single joint, with conflicting results depending on anatomic location. Although the mechanical impact of obesity or overweight in metabolic syndrome-associated OA has been well documented, some epidemiologic studies show an association between overweight and

OA on non-weight bearing joints, such as the hand, suggesting the contribution of other systemic factors in the pathogenesis of the disease (Abella et al, 2014; Visser et al, 2015; Yusuf et al, 2010).

Recent studies propose that OA can be associated not only with metabolic syndrome but can be also linked to each component of metabolic syndrome separately (Sellam & Berenbaum, 2013; Zhuo et al, 2012)namely hypertension (Lawrence, 1975), dyslipidemia (Sturmer et al, 1998), and obesity (Lohmander et al, 2009). These associations occur independently of others OA risk factors, suggesting that these metabolic factors could independently promote cartilage damage(Sellam & Berenbaum, 2013). Type 2 Diabetes (T2D) is another component of the metabolic syndrome that has been associated with a higher prevalence of musculoskeletal conditions when compared to the general population (Ramchurn et al, 2009). Moreover, in the United States, one report found that 47,3% of diabetic patients have some form of arthritis (Centers for Disease & Prevention, 2013).

Among all other metabolic components, the particular impact of T2D in OA has garnered great interest among the scientific community, with many authors considering it the most prominent factor, introducing the notion of diabetes-associated OA(Berenbaum, 2011).

1.3.3 DIABETES-ASSOCIATED OSTEOARTHRITIS

1.3.3.1 DATA SUPPORTING DIABETES-OSTEOARTHRITIS LINK

The first evidence of an association between OA and diabetes was published in 1961 (Waine et al, 1961). In the following years interest among the scientific community grew and new data, stemming from differently designed studies, further supported this link.

In the Ulm OA study, diabetic patients were more likely to have bilateral OA than non-diabetics, suggesting an increased risk of OA in non-insulin dependent diabetes. However, after adjustment for potential confounders the differences were no longer statistically significant (Sturmer et al, 2001). Also, in the Rotterdam study a higher prevalence of OA was found but only in diabetic patients aged 55 to 62 years (Dahaghin et al, 2007).

As OA and T2D share common risk factors, namely aging, the independent contribution of T2D on OA is difficult to analyze but some studies seem suggest an independent role of diabetes in OA development. In fact, even after adjustment for other OA risk factors, an association between OA and diabetes was observed in a different study (Nieves-Plaza et al, 2013). Reviewing epidemiological and experimental data, Francis Berenbaum suggested strong evidence for an independent contribution of diabetes in OA onset and progression (Berenbaum, 2011). Moreover, even non-

diabetic women with increased fasting serum glucose concentration experienced knee structural changes, suggesting that structural changes can also occur below the “diabetic range” of fasting serum glucose levels (Davies-Tuck et al, 2012).

Using magnetic resonance imaging (MRI) markers of early cartilage degeneration (Mosher & Dardzinski, 2004), diabetes was associated with increased severity of cartilage defects and was able to predict cartilage loss (Dunn et al, 2004; Jungmann et al, 2013; Prasad et al, 2013).

Total arthroplasty is the final stage in OA treatment, and thus represents a more aggressive progression. Thus, some studies used the rates of arthroplasty as a measure of OA severity and concluded that diabetes accelerates OA progression and alters the prognosis for OA development, resulting in increased risk of total joint replacement. Indeed, Schett et al, in a study observing 927 patients across a 20 year follow-up, found that type 2 diabetes resulted in a twofold risk of severe OA necessitating arthroplasty - independently of age, Body Mass Index (BMI) and other potential confounders (Schett et al, 2013). This increased rate of joint arthroplasty among diabetic patients was also observed in two more studies (King et al, 2013; Martinez-Huedo et al, 2013). Moreover, arthroplasties were performed at a younger age in patients with diabetes compared to non-diabetic patients (King et al, 2013), and type 2 diabetic patients exhibited more and worst OA symptoms and more frequent signs of synovitis (Schett et al, 2013; Siviero et al, 2009).

Recently two more studies corroborate this association. A meta-analysis highlighted the high frequency of OA in diabetic patients (Louati et al, 2015). F Eymard et al. showed that T2D was a strong factor associated with radiographic progression of knee OA in males. They also found diabetes as the only independent risk factor for knee OA out of all the components of metabolic syndrome (obesity, hypertension and dyslipidemia) in males (Eymard et al, 2015).

On the other hand, only one study failed to show a positive association between clinical OA and non-insulin dependent diabetes (Frey et al, 1996).

Different methodological approaches and study designs were used across the several studies mentioned. In fact, there are wide discrepancies among them, including in disease definition, measurements used to assess OA or diabetes, or anatomical location of OA considered. Also, a selection bias may be present since in many studies only patients with advanced or well-recognized disease were enrolled, ignoring those undiagnosed or with silent metabolic disease. On the contrary, supposedly healthy patients enrolled in control groups could also be undiagnosed. One should also always remain aware of the difficulty to ascertain a true independent association between diseases that share many common risk factors. Regardless of all this, a high frequency of OA is present in

diabetic patients and several independently conducted studies point to a likely association between both conditions.

Since OA is no longer a disease that only affects articular cartilage but impacts all joint tissues, the negative effect of diabetes in other articular tissues besides cartilage, has also been reported, further strengthening the association between both conditions. Effectively, as King K.B. and Rosenthal A.K. reviewed recently, diabetes-related tendon abnormalities and bone healing defects may also contribute to OA. For example, diabetic patients exhibited several alterations that could lead to bone stiffness (such as increased cortical porosity, excess mineralization, reduced subchondral bone heterogeneity, reduced levels of bone turnover biomarkers and elevated sclerostin levels) and consequently accelerate OA (King & Rosenthal, 2015; Yan & Li, 2013). The relation between diabetes and tendon alterations was also detected in one systematic review, although the authors argue that methodological limitations on most studies hamper the impact of the results (de Oliveira et al, 2011).

As described, there is some epidemiological data associating OA with diabetes and some changes in connective tissue physiology in diabetic patients are documented, but pathogenesis and causative factors for this association have not been fully elucidated yet. Some pathophysiological mechanisms explaining the high prevalence of OA in diabetes or how diabetic conditions contribute to the accelerated cartilage degradation have been proposed, however many other mechanisms can also be involved. Hopefully, the knowledge obtained from future experimental evidence may help explain the association or identify some potential mechanisms linking diabetes and OA.

1.3.3.2 Mechanisms involved in Diabetes-associated Osteoarthritis

Metabolic changes that occur in diabetes mellitus affect many organ systems, including articular cartilage. However, the specific impact of diabetes on the structural integrity of cartilage is not yet fully understood. Both OA and diabetes are chronic and debilitating diseases, whose increasing prevalence constitutes a global health problem. Also, OA and diabetes are complex diseases, with clinical heterogeneity and multifactorial etiologies, that share some risk factors. Since both are age-related diseases, most of the common mechanisms involved could also be a consequence of the aging process. Understanding the mechanisms by which these two diseases are interrelated can lead to the design of new preventive methods and therapeutic approaches that can halt both OA onset and progression.

Diabetes is a group of metabolic diseases characterized by chronic hyperglycemia as a result of defects in insulin secretion, action or both (American Diabetes, 2013). The prevalence of diabetes,

especially type 2, is increasing markedly across the world. As a consequence, diabetes is a growing global health problem that affected 382 million people in 2013. By 2035 this number is expected to increase to 592 million people, making it the most common metabolic disorder worldwide (Goldstein, 2003; Guariguata et al, 2014). Two pathogenic processes are involved in the development of T2D: resistance to the biologic action of insulin (or decreased insulin sensitivity), and impairment of insulin secretion from the pancreatic β-cells (American Diabetes, 2014; Goldstein, 2003; Iqbal, 2007). The abnormally elevated levels of circulating glucose promote a compensatory hyperplasia of β-cells. However, prolonged metabolic demand eventually overcomes this effect, resulting in pancreatic β-cell dysfunction and impaired insulin secretion. On top of impaired secretion, increased insulin resistance also contributes to insufficient insulin action. This deficient action inevitably results in an abnormal metabolism of carbohydrates, lipids and proteins (Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes et al, 2010).

As mentioned above, high glucose and insulin resistance are the main characteristics of T2D. Both have the potential to affect many of the key cell and matrix components of connective tissues and thus contribute to the degradative effects of diabetes in articular cartilage (Burner & Rosenthal, 2009). Several studies have tried to shed light on the mechanisms linking both diseases, and different hypothesis were advanced in recent years.

In chondrocytes, glucose is a major source of energy and is used in the synthesis of collagen and GAG, essential for PG synthesis, making it a vital nutrient for these cells (Lee & Urban, 1997; Mobasheri, 2012; Otte, 1991). Glucose reaches the articular cartilage by diffusion from the synovial fluid, where its concentration reflects that in the plasma. This means that conditions where glycemia is altered (such as diabetes) necessarily impact the glucose concentration that reaches chondrocytes (Maroudas, 1970; Otte, 1991). As glycolytic cells, chondrocytes need a regular supply of glucose to produce adenosine triphosphate (ATP) and maintain cell homeostasis. This makes chondrocytes particularly sensitive to altered levels of glucose (Mobasheri et al, 2008). In fact, high glucose can affect chondrocytes direct or indirectly. In the presence of high glucose, a decrease in the transport of dehydroascorbate in human chondrocytes was observed. Since dehydroascorbate is required for the synthesis of type II collagen, this decrease in transport is likely to compromise extracellular matrix production (McNulty et al, 2005). High glucose concentrations also induce a state of IGF-1 resistance in chondrocytes. IGF-1 has anabolic effects in articular cartilage, stimulating chondrocyte production of PGs, collagen type II and other ECM components. The resulting state of IGF-I resistance could thus limit the anabolic processes of cartilage (Kelley et al, 1999). This decrease in anabolic effects was confirmed in *in vivo* studies, where diabetic rats exhibited decreased levels of PGs and

remodeling of collagen in cartilage (Atayde et al, 2012). On the other hand, other studies show that high glucose can also favor inflammatory and catabolic responses in human chondrocytes. In fact, human OA chondrocytes exposed to high glucose concentrations presented higher mRNA levels of MMP-1 and -13, while in normal chondrocytes only MMP-1 mRNA levels were elevated (Rosa et al, 2011b). Moreover, high glucose was also reported to increase some critical inflammatory and catabolic mediators of OA, namely IL-1β, TNF-α, IL-6, inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2), which in turn translated into increased production of NO and prostaglandin E2 (PGE2), respectively, while collagen II was decreased (Chen et al, 2015) ; Rufino et al, 2014). Moreover, high glucose also seems able to induce Nuclear Factor-κB (NF-κB) activation (Rufino et al, 2014), a transcription factor that plays a central role in inducing the expression of inflammatory and catabolic genes, namely in chondrocytes and synovial cells (Liu-Bryan & Terkeltaub, 2015), which can explain its pro-inflammatory and pro-catabolic effects. Culture of both normal and OA human chondrocytes under high glucose also prevented TGF-β-induced downregulation of MMP-13 gene expression (Rosa et al, 2011b). Together these studies highlight the notion that high glucose directly affects chondrocytes, not only by promoting catabolic responses, but also by altering the response to anabolic factors (Rosa et al, 2011b). On the other hand, a down-regulation of perosixome proliferator-activated receptor γ (PPARγ) expression was identified as another mechanism underlying some of the inflammatory and catabolic responses described above that were effectively prevented by treatment with a PPARγ agonist (Chen et al, 2015). The anti-inflammatory and chondroprotective role of PPARγ in articular cartilage was previously demonstrated by the ability of its agonists to inhibit IL-1β induced production of MMP-13 and NO (Fahmi et al, 2001) and to counteract IL-1β-induced decrease in PG synthesis (Bordji et al, 2000) *in vitro*. These effects were also confirmed in *in vivo* studies (Kobayashi et al, 2005). Together, these studies suggest that impairment of anti-inflammatory pathways, such as down-regulation of PPAR-γ, can also mediate the effects of high-glucose in articular cartilage (Chen et al, 2015).

Furthermore, it is well established that a state of systemic low-grade chronic inflammation is present in type 2 diabetes and the association between inflammation and articular cartilage degradation has also been documented. In 2010, Stannus *et al* demonstrated that the loss of articular cartilage is associated with the circulating levels of IL-6 and TNF-α, suggesting that low grade inflammation participates in OA pathogenesis (Stannus et al, 2010). Moreover, human cartilage explants from OA-T2D patients were more reactive to pro-inflammatory stress (IL-1β) compared to cartilage explants from OA non-diabetic patients, showing an increase in IL-6 and PGE2 production. Likewise, in response to IL-1β, *in vitro* chondrocytes exposed to high glucose conditions, exhibited an increase in mRNA expression and protein release of inflammatory mediators (IL-6, COX2, PGE2), and

an increase in ROS and NO production, compared to normal glucose conditions. Additionally, when a glucose transport inhibitor, an inhibitor of the polyol pathway, a mitochondrial ROS scavenger or an inhibitor of NO -synthase were used, IL-6 production induced by IL-1β was reduced. Therefore, the increased reactivity of diabetic cartilage to pro-inflammatory stress can be a consequence of the interaction between glucotoxicity and IL-1β stress, responsible for perpetuating inflammation in chondrocyte (Laiguillon et al, 2015).

In addition, high glucose concentrations can saturate the glycolytic pathway, activating the secondary pathways of glucose metabolism that include the polyol, protein kinase C and hexosamine pathways. This will result in reactive oxygen species (ROS) production and oxidative stress, contributing to the glucotoxicity observed in diabetes (Brownlee, 2001; Obrosova, 2005; Yang et al, 2011). In chondrocytes, glucose uptake is mediated by glucose transporters (GLUTs), making them responsible for maintaining the normal glucose levels needed to preserve cartilage. In response to high glucose concentrations, human healthy chondrocytes are able to adjust glucose incorporation by downregulating one of the main transporters in chondrocytes, GLUT-1. However, OA chondrocytes lack the capacity to downregulate GLUT-1 and become unable to adapt to extracellular conditions, resulting in glucose accumulation (Rosa et al, 2009). Moreover, OA chondrocytes also exhibit a decrease in the expression of three enzymes that play an important role in glycolysis - α-enolase, glyceraldehyde 3-phoshate dehydrogenase and fructose biphosphate aldolase A - resulting in glucose accumulation (Ruiz-Romero et al, 2008). When normal and OA chondrocytes are exposed to a high glucose environment both show an initial increase in ROS production. However, only normal chondrocytes have the ability to restore ROS production to control levels, accompanying the decrease in GLUT-1 protein content, while OA chondrocytes sustain the increase in ROS production for long periods, leading to oxidative stress (Rosa et al, 2009). In fact, ROS accumulation was identified as a major mediator in cartilage degradation (Henrotin et al, 2003). It is involved in some processes such as reducing the synthesis of ECM components (Tiku et al, 1999), and it may also induce apoptosis (Del Carlo & Loeser, 2002). Moreover, ROS also mediate IL-1-induced activation of transcription factors such as NF-κB (Mendes et al, 2003a) and Activator Protein-1 (AP-1) (Mendes et al, 2003b), that are involved in degradation through activation of MMP gene expression (Fan et al, 2006; Liacini et al, 2002; Lo et al, 1998), and inflammation of articular cartilage (Goldring & Goldring, 2007; Marcu et al, 2010).

The accumulation of sorbitol observed in diabetic patients as a consequence of the activation of the polyol pathway likely contributes to diabetic complications by an increase not only in oxidative stress but also in cellular osmotic stress (Dvornik et al, 1973; Obrosova, 2005; Zhang et al, 2012). In

intervertebral disc cartilage (IVD) from diabetic rats this pathway appears to be increased and can promote cartilage degradation via p38 MAPK activation. However, since IVD is different from articular cartilage, it is yet to be proven whether this also occurs in articular cartilage and is related or not with OA (Cheng et al, 2013).

Furthermore, indirect effects of high glucose in articular cartilage can also be associated with the production of Advanced Glycation End Products (AGEs). AGEs are a heterogeneous group of compounds that result from the spontaneous reaction of reducing sugars with proteins. AGEs can only be removed from the body when the protein involved is removed, accumulating preferentially in proteins with slow turnover. The long half-life of collagen type II (more than 100 years) makes articular cartilage particularly sensitive to AGE accumulation (Verzijl et al, 2000b). AGE production and accumulation is known to play a critical role in the development of diabetes-related complications (Brownlee, 1992; Vlassara & Palace, 2002), and its accumulation in collagen was the first mechanism suggested for the association between diabetes and OA (Rosenbloom & Silverstein, 1996). In fact, a recent study showed that the levels of pentosidine, an AGE biomarker, were higher in the bone and cartilage of diabetic patients than in non-diabetic patients, at the time of joint replacement (Oren et al, 2011). Moreover, AGE accumulation has also been suggested as one of the mechanisms responsible for the age-related increase of OA incidence (Bank et al, 1998; DeGroot, 2004; DeGroot et al, 2004). The importance of AGEs in OA, through their role in the amplification of cartilage degradation, is well established. In chondrocytes, AGEs bind and activate their specific receptor, RAGE (Steenvoorden et al, 2006), inducing catabolic and inflammatory responses, and also induce chondrocyte hypertrophy, contributing to OA progression (Cecil et al, 2005). In fact, AGEs increase catabolic activity in chondrocytes by stimulating MMP-1, -3, -13, and TNF-α production (Loeser et al, 2005; Nah et al, 2007; Steenvoorden et al, 2006), and by enhancing inflammatory responses in OA chondrocytes by increasing PGE2 and NO levels via Mitogen-Activated Protein Kinase (MAPK) and NF-κB pathways, respectively (Huang et al, 2009; Nah et al, 2008). Moreover, AGEs can also disturb articular cartilage by affecting ECM components. Indeed, AGEs are able to reduce PG synthesis and increase the stiffness of the collagen network, eventually altering cartilage properties, such as the ability to resist to mechanical stress (DeGroot et al, 2001; Verzijl et al, 2002). Furthermore, the induction of chondrocyte apoptosis by AGEs was also reported (Yamabe et al, 2013). Together, these studies explain how AGE accumulation can be a factor that predisposes cartilage from diabetic patients to damage, possibly resulting in OA onset. Despite the evident effects of AGEs in articular cartilage, the independent role of AGEs in OA remains elusive and cannot be interpreted without considering the overall diabetic background (Vos et al, 2012; Willett et al, 2012). Therefore, accumulation of AGEs in cartilage occurs due to a synergistic effect of both diabetes,

through increased glucose availability, and aging, through prolonged accumulation over time (DeGroot et al, 1999; Verzijl et al, 2000a). Regardless of the known accumulation of AGE in diabetes, it remains important to understand whether this accumulation is by itself sufficient to trigger or worsen OA.

Additional mechanisms have also been proposed however they have not yet been completely understood. Hyperinsulinemia is another hallmark of T2D. Most often it occurs as a compensatory response to hyperglycemia caused by peripheral insulin resistance in the early stages of T2D, or in long term insulin therapy often needed in T2D, besides always required in type 1 diabetes. Chondrocytes express insulin receptors, making them insulin-sensitive cells (Rosa et al, 2011a). In articular cartilage, some studies show that insulin induce anabolic effects, like increased collagen II and PG synthesis, and inhibit catabolic responses like aggrecanase activity, NO production and IL-1β induced detrimental effects (Cai et al, 2002; Claassen et al, 2006). Recently, it was proposed that the insulin resistance observed in the synovium of T2D patients, may constitute a possible explanation for the increased susceptibility to trigger and accelerate OA in those patients. It was shown that a high insulin concentration was able to decrease cartilage damage in response to TNF-α, identified in the same study as a major player in T2D-associated OA. This effect suggests a protective and anti-inflammatory role for high insulin concentrations in fibroblast-like synoviocytes (FLS). Therefore, insulin resistance in the synovium would impair the protective role of high insulin concentration against TNF-α-induced cartilage degradation, likely constituting a possible mechanism involved in T2D- accelerated OA (Hamada et al, 2016). However, studies in other cell types reveal that hyperinsulinemia may have negative effects. For instance, hyperinsulinemia can contribute to retinopathy progression and it also has an adverse effect in lung structure and function, thus affecting respiratory health (Roysarkar et al, 1993; Singh et al, 2016; Wu et al, 2011). Furthermore, recent evidence suggests a possible contribution of hyperinsulinemia to induce inflammatory responses. In fact, high insulin concentrations induce NF-κβ activation in cardiac myoblasts (Madonna et al, 2014) and, according to unpublished results from our group the same seems to happen in chondrocytes. Moreover, higher levels of insulin were observed in overweight OA patients compared to overweight non-OA patients, supporting the idea that high concentrations of insulin can be involved in OA pathogenesis (Silveri et al, 1994). Therefore, the exact role of hyperinsulinemia in modulating chondrocyte functions and cartilage integrity needs to be further elucidated.

Recently, mitochondrial dysfunction has emerged as another potential link between diabetes and OA. Mitochondrial dysfunction is considered a major contributor for T2D development (Schrauwen & Hesselink, 2004), and is also considered a feature of OA pathogenesis (Blanco et al,

2011). In fact, OA articular chondrocytes showed a reduction in activity in mitochondrial complexes II and III, compared with cells from normal cartilage (Maneiro et al, 2003). According to previous studies, mitochondrial dysfunction increases MMP expression in normal human chondrocytes (Cillero-Pastor et al, 2013) and is also responsible for the amplification of chondrocyte inflammation induced by pro-inflammatory cytokines, through ROS production and NF-κB activation (Vaamonde-Garcia et al, 2012). In OA chondrocytes, pro-inflammatory cytokines are especially capable of inducing mitochondrial dysfunction by both inducing damage to mitochondrial DNA and by decreasing energy production and mitochondrial transcription, which induce apoptosis (Kim et al, 2010). Moreover, there is evidence of a metabolic cooperation and cross-talk between mitochondria and peroxisomes (Schrader & Yoon, 2007), implying that peroxisome dysfunction, as well as mitochondria dysfunction, could also lead to OA. In fact, a recent study showed differences in peroxisomal gene expression profile in OA chondrocytes from diabetic patients. This suggests the possibility that peroxisomal dysfunction in diabetic patients can play an important role in diabetes-associated OA (Kim et al, 2014).

Imbalances in mechanisms such as apoptosis and autophagy may also contribute to chondrocyte dysregulation and accelerated cartilage degradation observed in diabetes. The contribution of apoptosis to OA pathogenesis is difficult to evaluate since apoptosis can be both the inducer of cartilage degradation or a consequence of that same process. However, it is clear that increased apoptosis occurs in OA cartilage (Hwang & Kim, 2015). Comparing diabetic and non-diabetic animals, the former displayed an increase in apoptosis in nucleus pulposus (NP) cells, leading to early intervertebral disc degeneration (Jiang et al, 2013; Won et al, 2009), and in chondrocytes, during fracture healing (Kayal et al, 2009). These data indirectly suggest that apoptosis can also be a mechanism linking diabetes and OA. Autophagy and its role as another mechanism implicated in this association will be developed in the next sections.

1.4 AUTOPHAGY

To maintain homeostasis, cells need to constantly adapt to environmental cues, by balancing between biosynthetic and degradative processes. In eukaryotic cells, two main degradative processes can occur: proteasomal degradation, responsible for the breakdown of short-lived proteins; and autophagy, responsible for the degradation of long-live proteins, protein aggregates, and entire organelles (Meijer & Codogno, 2009).

In the last years, we have witnessed an explosion of research on a cell biology pathway called autophagy, or from the Greek roots “self-eating” process. Research on this process in yeast started

many decades ago, however, in mammals, it has been the subject of research since the 1960´s, with considerably more attention in recent years. It is an evolutionarily conserved intracellular process that occurs in all eukaryotic cells, with similar basic components shared between yeast and mammals (Glick et al, 2010).

Autophagy occurs constitutively and has two main functions: quality control, through the removal and degradation of damaged intracellular components, and maintenance of the cellular energetic balance, or cellular homeostasis, through the release of nutrients from macromolecules. However, additional functions have been suggested, including cellular remodeling and tissue differentiation, cellular survival and response to stress, cell death, senescence, and anti-aging functions. Indeed, autophagy is considered essential in many physiological and pathological conditions (Cuervo & Macian, 2012; Ravikumar et al, 2010).

Until now three basic forms of autophagy have been identified - macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). All of them promote proteolytic degradation of cytosolic components at the lysosome. However, they display different mechanisms for the delivery of the cytoplasmic material to lysosomes. Moreover, micro and macroautophagy have the capacity to engulf large structures, such as entire organelles, through selective and non-selective mechanisms. On the other hand, CMA can only degrade soluble proteins in a selective manner (Cuervo & Macian, 2012; Glick et al, 2010).

Macroautophagy is the most intensively studied type of autophagy and, due to increased interest in macroautophagy in articular cartilage; this thesis will focus solely on macroautophagy, hereafter referred to as “autophagy”.

1.4.1 AUTOPHAGIC PATHWAY

Autophagy is the main regulated cellular pathway responsible for the recycling of cell components and takes place as a dynamic process with several sequential steps: initiation, nucleation, elongation, maturation and degradation. The process begins with a double membrane structure, called the phagophore or isolation membrane. The phagophore elongates and engulfs intracellular cargo, resulting in the formation of a double membrane structure named the autophagosome. Subsequently, the outer membrane of the autophagosome fuses with the lysosome to form the autolysosome. In here, the degradation of the autophagosomal contents and of the inner membrane of the autophagosome occurs, by lysosomal acid proteases (Abounit et al, 2012). Proton pumps, such as vacuolar H^+ATPases, present in the lysosomal membranes, are responsible for the acidification of the autophagolysosome content. This acidification is required for the activation of

lysosomal enzymes responsible for content degradation (Yoshimori et al, 1991). Then, amino acids and other products generated from the degradation are transported back to the cytoplasm through membrane permeases, where they can be re-used for biosynthesis or energy production (Yang et al, 2006). The basic steps of autophagy are presented in figure 1.5.

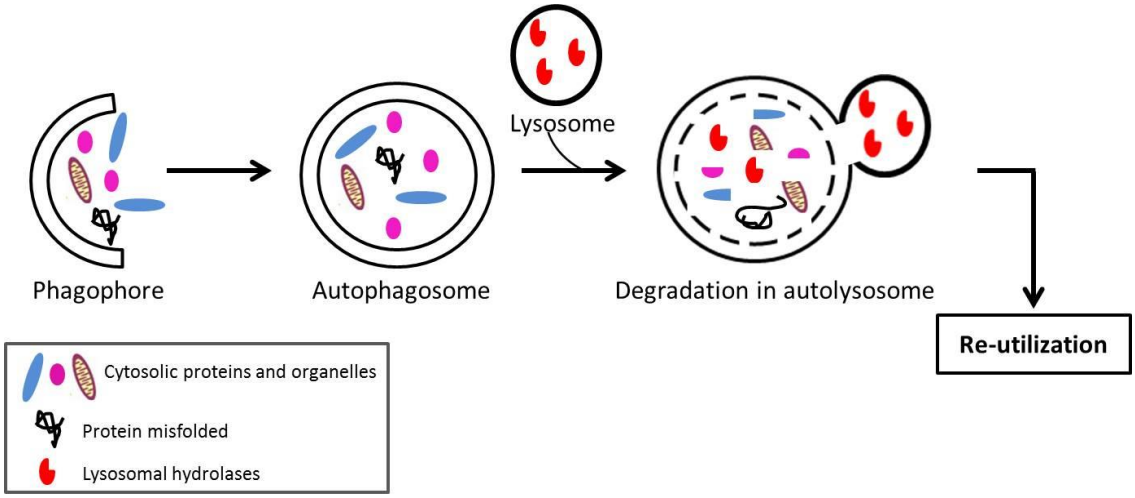

Figure 1. 5 - **Basic steps of the autophagic pathway.** First occurs the formation of the autophagosome that subsequently fuses with the lysosome to form the autolysosome. In the autolysosome, the degradation of the cytoplasmic material occurs by lysosomal hydrolases. The resulting degraded products are released and can be re-utilized.

Autophagosome formation is the rate-limiting step of this process and involves three major steps, namely, initiation, nucleation and elongation/enclosure (Meijer & Codogno, 2009). These steps are energy dependent and are regulated by autophagy-related proteins (Atg). Most Atg proteins were originally identified and characterized in yeast but subsequent studies demonstrated that they remain highly conserved in higher eukaryotes (Mizushima et al, 2011):

- The initiation step is controlled by a stable ULK complex formed by the Uncoordinated-51-Like Kinase (ULK 1/2), mammalian Atg13 (mAtg13), Focal Adhesion Kinase (FAK) Family Interacting Protein of 200kDa (FIP200), and Atg101. This complex is essential in the induction of autophagosome formation. It is the terminal target of various signaling cascades that regulate autophagy, such as mammalian target of rapamycin (mTOR) and AMP-activated kinase (AMPK) pathways, which means that autophagy initiation is sensitive to growth factor, energy and nutrient availability. ULK kinases stimulation occurs through reactions of phosphorylation and dephosphorylation that occurs in the various subunits of the ULK complex (Mizushima, 2010; Reggiori et al, 2012).
- The nucleation step involves class III phosphatidylinositol 3-kinase (PI3K) (Vacuolar protein sorting protein 34, hVps34) that associates with Beclin 1 and p150 to form the class III PI3K complex (Funderburk et al, 2010). The recruitment of this complex to the phagophore requires ULK1

kinase. The interaction between Beclin-1 and hVps34 promotes its catalytic activity and leads to the production of phosphatidylinositol-3-phosphate (PtdIns(3)P or PI3P) in mammals, through phosphorylation of phosphatidylinositol (PtdIns). PtdIns(3)P is then essential to phagophore elongation and to recruit components essential for autophagosome formation (Mizushima et al, 2011; Petiot et al, 2000).

- In the elongation/enclosure step, the final step of autophagosome formation, two ubiquitin-like conjugation systems are required, Atg12 and microtubule-associated protein 1 (MAP1) light chain 3 (LC3):

- The first one requires the conjugation of Atg12-Atg5, which is produced by two ligases, Atg7 (homologous to E1 ubiquitin-activating enzyme) and Atg10 (homologous to E2 ubiquitin-conjugating enzyme). Conjugated Atg12-Atg5 then associates with Atg16L dimers to form a multimeric Atg12-Atg5-Atg16L complex, which associates with the phagophore membrane (Mizushima et al, 2003; Reggiori et al, 2012). This complex is present on the outer side of the isolation membrane and is essential for its proper elongation and dissociates from the membrane immediately before or after autophagosome formation (Mizushima et al, 2011).

- The second one requires the cleavage of the C-terminus of the unprocessed form of LC3 (pro-LC3) (a ubiquitin like protein that is the mammalian homologue of the autophagy-regulated Atg8 in yeast) by Atg4 to produce the soluble form LC3-I, or cytosolic LC3. LC3-I subsequently conjugates with phosphatidylethanolamine (PE), with the participation of Atg7 and Atg3 (another E2-like enzyme). The resulting lipidated form of LC3, LC3-II, associates with newly forming autophagosome membranes and is present in both the inner and outer membranes of the autophagosome. This conversion of LC3-I to LC3-II is a key regulatory step in autophagosome formation (Reggiori et al, 2012). LC3-II allows the expansion and closure of the autophagic vacuole which means it is required for autophagosome formation and is commonly used as a marker for autophagosomes (Nakatogawa et al, 2007). Autophagy has been considered a nonselective degradation process that engulfs cytosolic components in a random way. However, in the last years accumulating evidence identified selective forms of autophagy responsible for degrading specific organelles and protein aggregates. It is proposed that LC3-II can recruit adaptor molecules, such as p62 and neighbor of BRCA1 gene (NBR1) to autophagosomes via the LC3-interacting region (LIR) and thus promote selective cargo incorporation into the autophagosome (Lamark et al, 2009). LC3-II in the outer membrane of the autophagosome is removed by Atg4 that cleaves the PE, returning it to the cytosol for reuse. LC3-II in the inner membrane is digested with the cargo. To our knowledge,

LC3-II is the only protein known to be associated with a completed autophagosome (Kabeya et al, 2000).

These two conjugation systems seem to be interconnected. The Atg12-Atg5-Atg16L complex plays an essential role in Atg8/LC3-PE conjugation, and has an E3(ubiquitin ligase)-like activity for LC3 lipidation, contributing to the expansion of the autophagosomal membrane and to the correct localization of LC3 lipidation (Fujita et al, 2008; Geng & Klionsky, 2008; Hanada et al, 2007). Atg8/LC3 appears to be essential for the function of the Atg12 conjugation system (Sou et al, 2008). Any disruption in these complexes may lead to dysfunctional autophagy. A schematic representation of autophagosome formation and its regulation are presented in figure 1.6.

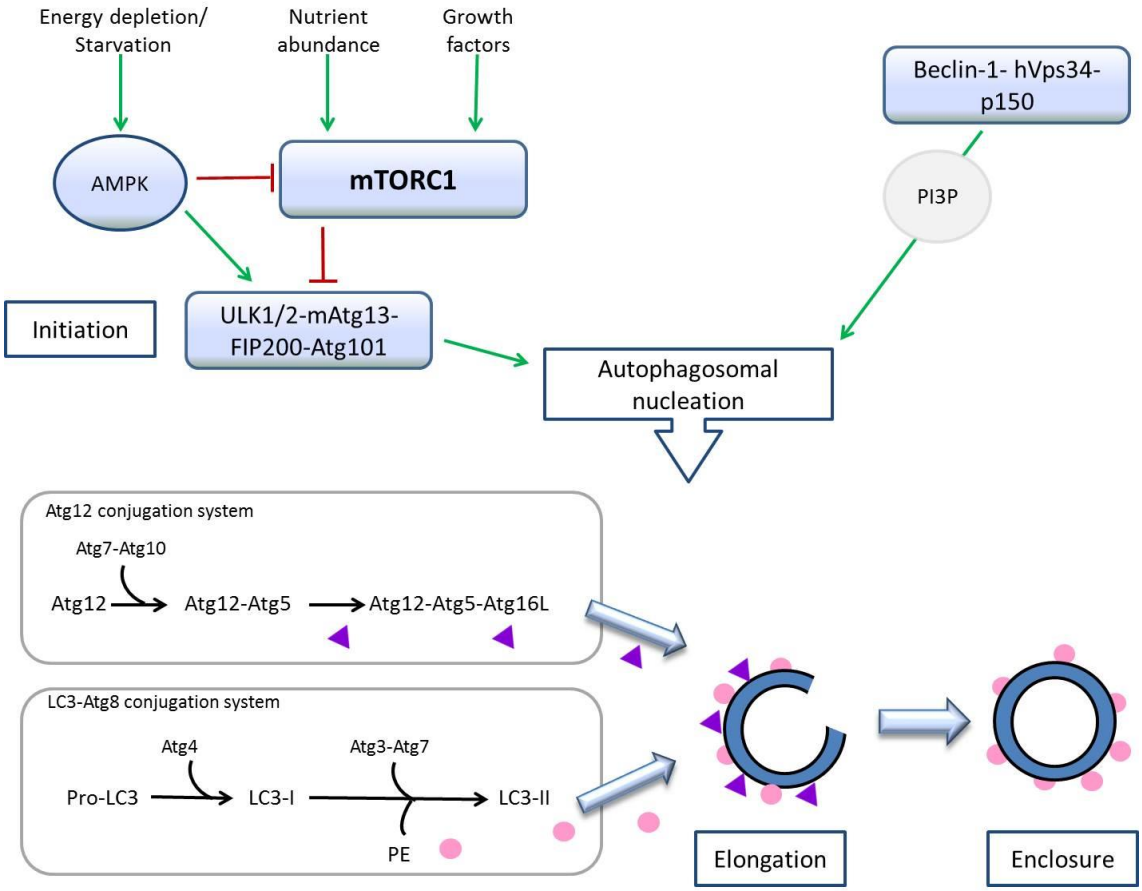

Figure 1. 6 - **Schematic representation of the rate-limiting step of the autophagic pathway: the autophagosome formation.** The autophagosome formation involves initiation, nucleation, and elongation/enclosure steps that are regulated by different Atgs. The ULK complex mediates the initiation step. The nucleation step is regulated by class III PI3K complex. Finally, the elongation and enclosure requires two ubiquitin-like conjugation systems, the Atg12-Atg5 complex and LC3-II. (Green arrows represent activation, whereas red lines represent inhibition). Adapted from (Choi et al, 2013).

1.4.2 SIGNALING PATHWAYS INVOLVED IN AUTOPHAGY REGULATION: THE MAMMALIAN TARGET OF RAPAMYCIN (mTOR) PATHWAY

Autophagy is a constitutive process that can be deleterious when present in insufficient or excessive levels. Thus, the tight regulation of autophagy is crucial and this dynamic regulation is one of its characteristic features. Accordingly, autophagy can be stimulated by physiological stress conditions (such as starvation, hypoxia, and energy depletion), hormonal stimulation, pharmacological agents, innate immune signals and by some pathological conditions (Mizushima et al, 2010). Autophagy is controlled by multiple upstream signaling pathways, most of which converge on mTOR. Although this pathway is traditionally considered the main step involved in autophagy regulation, other mTOR independent pathways are also involved (Ravikumar et al, 2010).

The mTOR, the target of the natural compound rapamycin, is a highly conserved serine/threonine kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family (Hung et al, 2012). mTOR is a central regulator of cell growth, proliferation and survival. It is also a major regulator of autophagy in mammalian cells and is composed of two functionally distinct complexes, mTORC1 and mTORC2, with both shared and unique subunits. Both complexes share the catalytic mTOR subunit, mammalian lethal with sec13 protein 8 (mLST8, also known as GβL), DEP domain containing mTOR-interacting protein (DEPTOR), and Tti1/Tel2 complex. Regulatory-associated protein of mammalian target of rapamycin (raptor) and proline-rich Akt substrate 40kDa (PRAS40) are unique subunits present in mTORC1. Rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated map kinase-interacting protein 1 (mSin1) and protein observed with rictor 1 and 2 (protor1/2) are specific of mTORC2 (Laplante & Sabatini, 2012). One difference between them is the sensitivity to rapamycin: mTORC1 is rapamycin sensitive while mTORC2 is insensitive. Rapamycin interacts with FK506-binding protein 12 (FKBP12) and this complex directly binds to mTORC1, causing its inhibition. Exactly how this binding inhibits mTORC1 remains unknown (Benjamin et al, 2011). Although normally insensitive to rapamycin, depending on the cell type, mTORC2 can become sensitive to rapamycin after prolonged treatment (Sarbassov et al, 2006).

mTORC1 is the best characterized mTOR complex, responsible for the control of many processes. In fact, it is responsible for the stimulation of anabolic processes such as protein and lipid synthesis, ribosome biogenesis, gene transcription, and metabolism, and also inhibits catabolic processes such as autophagy (Albert & Hall, 2015; Hung et al, 2012). Effectively, mTORC1 is highly sensitive to cellular nutrient content. In high nutrient and energy conditions, mTORC1 is activated downstream of growth factor receptor signaling, involving activation of Akt and PI3Kinases, and promotes cell growth through induction of ribosomal protein expression and increased protein translation. In low nutrient and energy conditions mTORC1 is inactivated, resulting in diminished cell growth and allowing the restauration of energy and nutrient levels by autophagy activation (Benjamin et al, 2011).

In turn, mTORC2 signaling controls members of the AGC kinase family including Akt, serum/glucocorticoid-induced protein kinase1 (SGK1) and protein kinase C (PKC), thus playing an important role in actin cytoskeletal organization, and cell survival and metabolism. Although it seems that mTORC2 is insensitive to nutrients, it responds to growth factors, although the exact mechanism is still unknown (Benjamin et al, 2011; Laplante & Sabatini, 2012).

1.4.2.1 UPSTREAM REGULATORS OF mTORC1

mTORC1 is upstream regulated by at least five major cues: growth factors, energy status, oxygen levels, nutrient availability, and other stress signals, such as DNA damage. Growth factors regulate mTOR activity through the PI3K/Akt signaling pathway or through the stimulation of the mitogen-activated protein kinase (MAPK) ERK1/2 and the p90 ribosomal S6 kinase (RSK), all inhibiting the Tuberous Sclerosis Complex (TSC1/TSC2) complex. TSC2 acts as a GTPase-activating protein (GAP) for the small GTPase Ras homolog enriched in brain (Rheb), that in conjunction with TSC1 form the TSC1/TSC2 complex. This complex promotes the conversion of Rheb-GTP (active form) to Rheb-GDP (inactive form). The Rheb-GTP interacts with mTORC1 and stimulates its kinase activity. Thus, growth factors inhibit the TSC1/TSC2 complex, allowing Rheb-GTP to activate mTOR. Regarding the energy status and oxygen levels, decreased ATP levels and hypoxia, through activation of AMPK and regulated in development and DNA damage responses 1 (REDD 1), activate TSC1/TSC2 complex. This complex activation increase Rheb-GDP, thus inhibiting mTORC1. Other stress signals, such as DNA damage, also inhibit mTORC1 via AMPK-TSC2 signaling pathway (Hung et al, 2012; Laplante & Sabatini, 2012; Wullschleger et al, 2006). The mechanism by which mTORC1 senses amino acids is poorly understood and requires further studies, but it has been proposed that amino acids regulate mTORC1 signaling through a pathway requiring Rheb-dependent activation of the complex and a second family of GTPases, the Rag proteins (Sancak et al, 2010; Sancak & Sabatini, 2009).

1.4.2.2 DOWNSTREAM REGULATORS OF mTORC1

To date, only a few mTORC1 substrates were identified. Ribosomal protein S6 kinase1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), which regulate protein synthesis and promote messenger RNA translation, and ULK complex that regulates autophagy, are best characterized substrates of mTORC1. 4E-BP1 represses mRNA translation. Upon phosphorylation by mTORC1, 4E-BP1 dissociates from eIF4E. This promotes the assembly of the eIF4F complex which is required for cap-dependent translation initiation. Upon phosphorylation by mTORC1, S6K1 phosphorylates multiple substrates such as ribosomal protein S6, eIF4B and programmed cell death 4 (PDCD 4), involved in mRNA maturation and protein translation. Therefore, activation of S6K and inhibition of 4EBP1 by mTOR promotes translation and cell growth (Albert & Hall, 2015; Wullschleger et al, 2006). Regarding autophagy, under nutrient-rich conditions, mTORC1 associates with the ULK complex, and phosphorylates ULK1 and mAtg13. This phosphorylation suppresses ULK1 kinase activity and consequently reduces the formation of autophagic vesicles. On the other hand, under starvation conditions, mTORC1 is inactivated and dissociates from the ULK complex which results in ULK1 dephosphorylation leading to autophagy induction (Hosokawa et al,

2009; Mizushima, 2010; Mizushima et al, 2011). However, this interaction between mTOR and ULK complex may not fully explain the autophagy activation. Other sensors, namely AMPK may also be required for ULK regulated autophagy activation (Kim & Guan, 2011; Kim et al, 2011). A schematic diagram of the mTORC1 pathway is presented in figure 1.7.

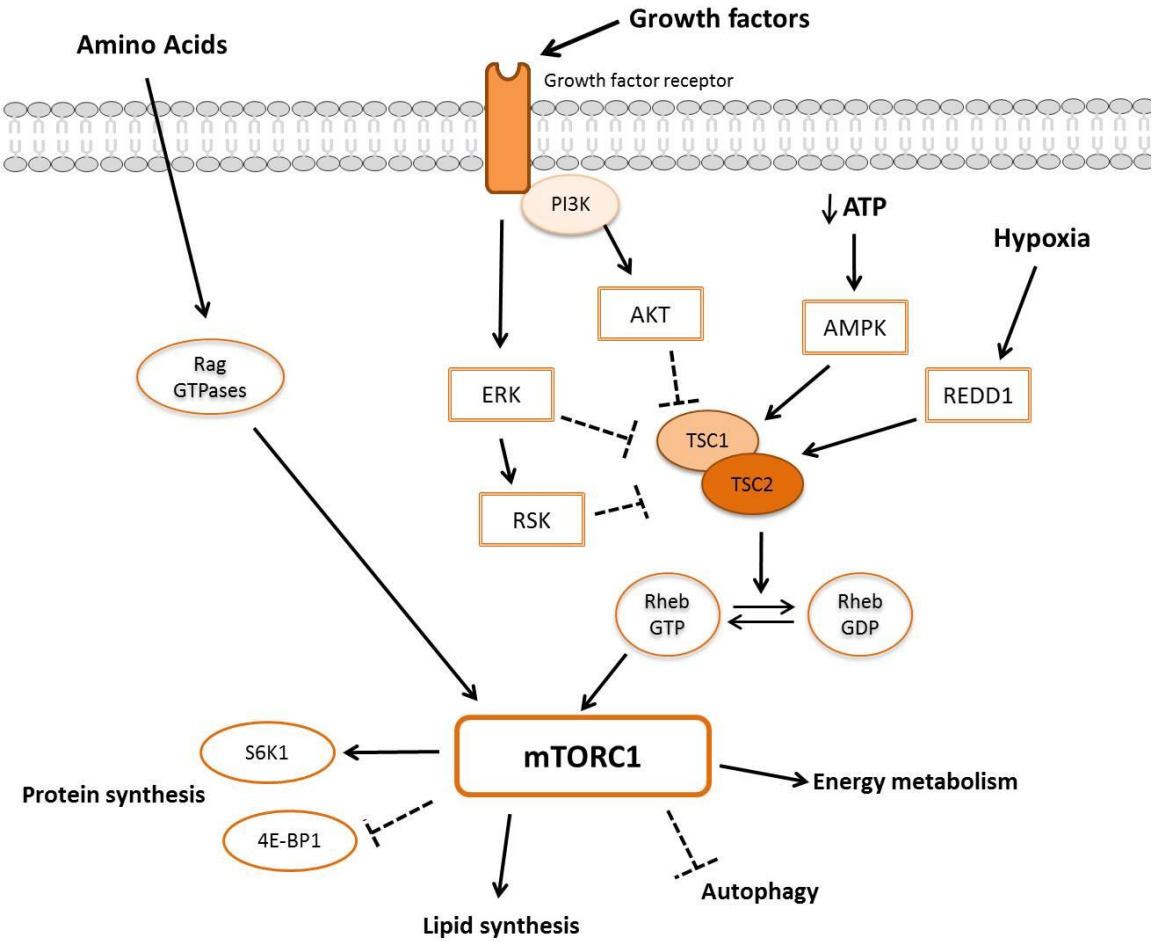

Figure 1. 7 - **A schematic diagram of the mTORC1 signaling pathway.** (Arrows represent activation, whereas dashed lines represent inhibition)

Autophagy can also be regulated by mTOR independent signaling pathways. In fact, in nutrient rich conditions, Beclin-1 binds to B cell lymphoma 2 (Bcl-2), an antiapoptotic protein, which prevents the association of Beclin-1 with the class III PI3K, therefore suppressing autophagy. So, before stimulating autophagy, Beclin-1 has to dissociate from this inhibitory complex. In starvation conditions, Jun-N-terminal kinase 1 (JNK1) is responsible for Bcl-2 phosphorylation. This phosphorylation is essential for the disruption of the Bcl-2/Beclin complex allowing Beclin-1 to interact with class III PI3K and inducing autophagy. Other pathways that also regulate autophagy independently of mTORC1 include cAMP-Epac-PLC-ε, Ca^{2+}-calpain and inositol signaling pathways (Sarkar, 2013; Sarkar et al, 2009).

1.4.3 AUTOPHAGY AND DISEASE

Autophagy is an indispensable mechanism for all cell types. Growing evidence suggests that it plays a key role on several physiological functions, highlighting its possible impact on human health. In fact, it is not surprising that disruption of autophagy is associated with many human diseases, including diabetes, cancer, neurodegenerative disorders and osteoarthritis (Gonzalez et al, 2011; Lotz & Carames, 2011).

1.4.3.1 AUTOPHAGY AND DIABETES

As mentioned previously, autophagy is regulated by the cellular nutritional and energy status, therefore, its involvement in metabolic diseases is not surprising. Indeed, in diabetes, many studies report impaired autophagy in various tissues, including pancreatic beta cells (Masini et al, 2009). Moreover, altered autophagy can contribute to or worsen diabetic complications or, on the other hand, it can act as a protective response. In fact, diabetic conditions can increase or decrease autophagy in a tissue-dependent manner. For instance, the number of autophagosomes observed in diabetic adipocytes from obese T2D patients was higher than in non-diabetic, in accordance with attenuated mTOR activity, suggesting an increased autophagic activity in diabetic adipocytes (Ost et al, 2010). Also, nucleus pulposus of diabetic rats exhibited higher autophagy levels compared to control rats. This increased autophagy seems to be a protective mechanism against accelerated apoptosis and senescence, in order to prevent intervertebral disc degeneration (Jiang et al, 2013). On the opposite, in different cell types, diabetic conditions decreased autophagy. For example, in both mesangial cells and podocytes of diabetic animals, an increase in mTOR and decreased levels of Beclin-1, Atg12-Atg5 and LC3-II were detected. Furthermore, it was suggested that mTOR activation and defective autophagy can be involved in the development of diabetic nephropathy (Fang et al, 2013; Inoki et al, 2011; Mori et al, 2009). Similarly, hearts of diabetic animals showed decreased autophagic flux. However, in the case of type 1 diabetes this decreased autophagy seems to be an adaptive response to protect against cardiac injury, while in type 2 diabetes it seems to contribute to cardiac injury (Kobayashi & Liang, 2015). Therefore, it seems evident that autophagy is somehow altered in diabetes, although how it interacts with diabetic lesions appears to be a complex process.

1.4.3.2 AUTOPHAGY AND OSTEOARTHRITIS

Autophagy is increasingly recognized as an essential mechanism to preserve normal healthy articular cartilage. In fact, normal human cartilage expresses high levels of autophagy markers, including ULK1, Beclin1 and LC3-II, suggesting that autophagy is a constitutively active mechanism

present in cartilage. On the other hand, in human OA cartilage and in an experimental OA-induced model, the expression of autophagy markers appears reduced compared with normal cartilage (Carames et al, 2010; Zhang et al, 2015). In accordance with these findings, mTOR appears overexpressed in human OA cartilage, and mouse models of experimental OA, when compared with normal human/control cartilage (Zhang et al, 2015). Moreover, a positive correlation between gene expression of mTOR in peripheral blood mononuclear cells (PBMC) and articular cartilage was detected in end-stage OA patients (Tchetina et al, 2013).

In contrast with these findings, other studies reported an increase in LC3-II and Beclin1 expression in OA chondrocytes, implying an up-regulation of autophagy in OA compared to non-OA chondrocytes (Sasaki et al, 2012). Accordingly, another study found that OA tissues display numerous autophagic LC3 puncta while no elevation in punctate LC3 is observed in healthy tissues (Bohensky et al, 2009). The authors justify these opposite observations with the different location of harvested OA cartilage that potentially corresponds to different OA stages. In the study by Sasaki *et al.* (2012), cartilage obtained from lateral femoral condyles was identified as mild OA, while cartilage from medial femoral condyles was identified as severe OA. Autophagy markers were expressed differently between these two regions. Mild OA cartilage had a strong expression of autophagy markers compared with non-OA cartilage and severe OA cartilage. With these results, the authors proposed that during OA progression, autophagy can act as an adaptive response in an attempt to protect cells. However, once severe OA is established, a decrease in autophagy was detected, meaning that failure of autophagy could contribute to OA progression (Sasaki et al, 2012).

A similar dual behavior of autophagy can be seen when we look at its effect on cell death. Many studies consider autophagy as a protective mechanism that avoids cell death. In fact, autophagy activation protects against chondrocyte death (Carames et al, 2012a; Carames et al, 2012b; Sasaki et al, 2012), while reduction of autophagy markers is accompanied with increased apoptosis (Carames et al, 2010; Zhang et al, 2015). Moreover, up-regulation of autophagy also seems to be able to suppress glucocorticoid-stimulated chondrocyte apoptosis (Liu et al, 2014). However and paradoxically, autophagy may also constitute an alternative form of cell death (Levine & Yuan, 2005; Maiuri et al, 2007). Indeed, monosodium urate crystals seem to promote chondrocyte death through autophagy activation (Hwang et al, 2015). Recently, Chang J. *et al.* (2013) found that autophagy activation protected young chondrocytes from cell death but eventually lead to autophagic cell death in OA chondrocytes. These results propose a possible dual role for autophagy in chondrocytes in different stages of OA progression: it could have both a cytoprotective and a death-promoting role in OA pathogenesis (Chang et al, 2013). As part of OA pathogenesis, the death

of chondrocytes appears to be due to a complex interaction between autophagy and apoptosis, with cells exhibiting signs of both mechanisms across different stages of OA progression and different anatomical locations (Almonte-Becerril et al, 2010). These conflicting results suggest that autophagy could promote either chondrocyte survival or death depending on donor age, the presence and stage of OA, and the type of autophagy inducer (Hwang et al, 2015). In fact, in other cell types this dual role of autophagy was also reported, depending on the type of cellular stress present (Chen et al, 2010). Notwithstanding, this relation between autophagy and cell death is not fully understood, thus requiring more studies to define the exact interaction between them (Musumeci et al, 2015b).

Several studies report the protective role of autophagy under stress conditions. Both nutritional (serum-starved) and catabolic stresses [treatment with IL-1β or sodium nitroprusside (SNP)] increased the normal autophagic activity in chondrocytes (Sasaki et al, 2012). Mechanical stress was also tested and an increase in LC3-II levels was detected 24h after mechanical injury, while 48 and 96h after injury, LC3-II levels appear to decrease (Carames et al, 2012b). Similarly, the biomechanical dental stimulation that leads to degradation of the cartilage from the temporomandibular joint also increased autophagy as an early response (Zhang et al, 2013). Intermittent cyclic mechanical tension (ICMT) leads to calcification of end plates of the intervertebral disc, which is responsible for its degeneration. Short-term ICMT increased autophagy and it was accompanied by an insignificant calcification of end plate chondrocytes. However, long term ICMT suppressed autophagy, leading to endplate chondrocyte calcification. Although the cartilage of the intervertebral disc differs from articular cartilage, a similar mechanism may be involved in the latter (Xu, 2015; Xu et al, 2014). Furthermore, in response to induced mitochondrial dysfunction, chondrocytes showed an early increase in autophagy as a compensatory mechanism against cell stress. However, when prolonged stress exceeds cellular compensation, damage occurs (Lopez de Figueroa et al, 2015). These results are in agreement with the ones reported by Sasaki *et al*. 2012. Initially, mitochondrial dysfunction and mechanical injury activate autophagy as an early response to stress, but this is insufficient to protect cartilage and eventually a decrease in autophagy is observed. Moreover, the protective role of autophagy in OA was recently confirmed using a rabbit OA model, established by intra-articular injection of collagenase, where an autophagy inhibitor was injected. The group receiving collagenase and the autophagy inhibitor exhibited more histological changes in cartilage with higher Mankin scores compared to the group receiving only collagenase, suggesting that autophagy inhibition worsens experimental OA (Cheng et al, 2016). Together all these results provide strong evidence that autophagy has an important role in protecting chondrocytes from different stressors and, therefore, can be involved in OA pathogenesis.

As reported above, mTOR is one of the most important autophagy regulators. So, to understand the exact role of mTOR signaling in OA pathophysiology, *in vivo* studies using specific KO mice were performed. Inducible cartilage-specific mTOR KO mice were subjected to experimental OA induced by destabilizing the medial meniscus (DMM model) and an increase in the expression of autophagy markers (ULK1, AMPK1, Atg5, LC3) was observed, as expected. Furthermore, a protection from experimentally induced-OA was also observed, with a reduction in cartilage degradation, proteoglycan loss, loss of cellularity, and synovial fibrosis. Moreover, a decrease in apoptotic cells and catabolic factors, such as MMP-13 and MMP-induced type II collagen breakdown product C1,2C, both involved in OA, was also detected (Zhang et al, 2015). Several evidences suggest that PPARγ plays a protective role in articular cartilage. In fact, PPARγ KO mice exhibited increased apoptosis as well as production of inflammatory and catabolic factors, and decreased expression of anabolic factors, resulting in an accelerated OA phenotype (Vasheghani et al, 2013; Vasheghani et al, 2015). A significant reduction in LC3-II and an increase in mTOR expression were detected in those animals, compared with a control group. Furthermore, in PPAR-deficient OA chondrocytes, the restoration of PPAR expression was able to downregulate mTOR expression and to up-regulate LC3-II expression. Additionally, a decreased expression of catabolic and inflammatory factors and an increased expression of anabolic factors were also observed. On the other hand, in PPARγ-mTOR double KO mice, a significant protection from experimentally induced-OA (DMM model) was observed. These mice presented decreased cartilage degradation, proteoglycan loss and chondrocyte loss, as well as a significant reduction in the percentage of MMP-13 positive cells, and an increase in LC3-II expression. Thus, decreased PPARγ contributes to mTOR upregulation that is responsible for autophagy suppression, decrease in chondrocyte survival and increase in catabolic activity. Ultimately this will accelerate OA progression (Vasheghani et al, 2015). Both *in vivo* studies confirm that mTOR deletion has a protective role in OA, thus helping reinforce the role of decreased autophagy in OA development.

Autophagy, that also regulates inflammatory processes, can prevent cartilage damage by interfering with inflammation, an important feature of OA (Rahmati et al, 2016). In fact, some evidence suggests that activation of autophagy could prevent the induction of inflammatory responses (Salminen et al, 2012), including in the context of articular cartilage. According to Tchetina *et al.* (2013), increased mTOR expression in PBMC was associated with synovitis (Tchetina et al, 2013). This link between mTOR and inflammation was also demonstrated using an *in vivo* model of inflammatory arthritis where the inhibition of mTOR was able to reduce osteoclast numbers and activity, protect against local bone erosions and cartilage damage, and decrease synovitis (Cejka et al, 2010). The same association was observed in an *in vivo* model of OA where mTOR inhibition was able

to reduce synovial inflammation and decrease IL-1β expression in the synovium (Carames et al, 2012a). Moreover, chondrocytes from PPARγ KO mice showed an increase in the expression of inflammatory mediators (COX-2 and iNOS) associated with enhanced expression of mTOR and a decrease in autophagy markers (Vasheghani et al, 2015). On the other hand, when human OA chondrocytes were treated with IL-1β, an increased expression of mTOR was observed in conjunction with increased expression OA catabolic factors and a decreased expression of collagen type II, suggesting that pro-inflammatory cytokines can also alter mTOR expression during OA (Zhang et al, 2015). Viewed together, these studies suggest an important connection between mTOR, inflammation and cartilage damage.

It is well known that aging is one of the major risk factors for OA. Not all old people develop OA, however, age-related changes can precipitate OA onset (Lotz & Loeser, 2012). The low turnover of ECM and cells makes articular cartilage more sensitive to the accumulation of age-related changes. Age-related changes in articular cartilage include disturbed structural organization of ECM due to formation and accumulation of AGEs and cartilage calcification leading to increased fibrillation, responsible for alterations in cartilage properties. With respect to cellular age-related changes, those include cellular senescence, reduction in cartilage cellularity, mitochondrial dysfunction, and altered growth factors responsiveness. Alterations in protective mechanisms such as decreased antioxidant defense and reduced autophagy, also occur with aging, disturbing the anabolic-catabolic equilibrium and reducing the remodeling and repair ability of cartilage (Hui et al, 2016; Lotz & Loeser, 2012). In fact, more recently, autophagy has been studied as a mechanism that can regulate age-related changes in articular cartilage. Both aging human chondrocytes and old mice exhibit a reduction in constitutive autophagy. Moreover, findings suggest that compromised autophagy, as a consequence of aging, precedes the decrease in cartilage cellularity and the onset of structural damage. (Carames et al, 2015; Carames et al, 2010; Hui et al, 2016). Indeed, a reduced repair response, an altered homeostasis and a defective removal of damaged proteins, are important hallmarks of aged tissues (Cuervo, 2008; Cuervo et al, 2005). In this context, some mechanisms occurring during the aging process can explain the impaired autophagy observed, including defects in autophagy induction and inefficient lysosomal clearance. For instance, the altered hormonal regulation of autophagy observed in aged tissues can be a possible explanation. In fact, in old organisms the ability of glucagon to upregulate autophagy appears compromised (Cuervo et al, 2005). Moreover, signaling pathways involving pro-longevity factors such as Sirtuin 1 (SIRT1) and transcription factor forkhead-box O3 (FOXO3), and pro-senescence factors like mTOR, NF-kB and p53 are also autophagy regulators and thus can be involved in aging (Salminen & Kaarniranta, 2009). Furthermore, decreased autophagy can also be explained by the impaired capacity of the lysosomes to fuse with autophagosomes and

the failure of lysosomal hydrolases that decrease the proteolytic efficiency of lysosomes (Cuervo, 2008). In fact, the intralysosomal accumulation of undegradable materials that occur with aging hampers the lysosomal degradative capacity resulting in decreased autophagy (Brunk & Terman, 2002a; Brunk & Terman, 2002b; Terman & Brunk, 2004).

As expected by the decrease in autophagy with aging, an elevated mTOR activity in cardiac and skeletal muscle tissues in aged mice and humans, and in a mouse model of progeria has also been reported (Ramos et al, 2012; Sandri et al, 2013). Several studies in multiple organisms reported that mTOR inhibition extends lifespan, however, these mechanisms remain unclear (Lamming, 2016). Autophagy, regulated by mTOR, seems to be an important process that contributes for lifespan extension. mTOR inhibition derepresses autophagy that favors degradation of aberrant proteins and damaged organelles, thus protecting from toxicity, and consequently slowing aging. Additionally, other processes regulated by mTORC1 can also contribute to the pro-longevity effects of mTOR inhibition, including reduction of protein synthesis, regulation of mitochondrial function, reduction in inflammation, increased stress resistance and preserved stem-cell function (Johnson et al, 2013). Being aging and OA closely intertwined, it appears that autophagy can be a common mechanism involved in cartilage degradation in both conditions.

1.4.4 Pharmacological autophagy activation- a potential therapeutic target for Osteoarthritis

Although many advances have been made in understanding the pathophysiological processes of OA, to this moment no effective treatments to stop or prevent OA progression have been discovered. Few treatment options for OA are available, and they mainly focus on symptom relief and improving joint function. Currently, it relies in a combination of non-pharmacological and pharmacological approaches. Decisions made for OA treatments greatly depend on different patient-related factors, such as the occurrence of other co-morbidities and the presence of one or multiple OA affected joints. Thus, different treatment guidelines have been proposed according to the recommendations from scientific authorities including The European League Against Rheumatism (EULAR), the American College of Rheumatology (ACR), the Osteoarthritis Research Society International (OARSI), the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO), and the American Academy of Orthopaedic Surgeons (AAOS) (Brown, 2013; Bruyere et al, 2014; Bruyere et al, 2016; Hochberg et al, 2012; McAlindon et al, 2014; Zhang & Doherty, 2006). The non-pharmacological options include patient education, weight management and exercise programs. With respect to pharmacological options, these include analgesic and anti-inflammatory agents (non-selective or selective COX-2 inhibitors), Symptomatic Slow-Acting Drugs for Osteoarthritis (SYSADOA) and intra-articular corticosteroids injection. When the goals are not

achieved with conservative treatment and the disease has progressed to a severe clinical stage resulting in poor quality of life, surgical options should be considered**.** However, one must always bear in mind the considerable costs, risks and long-term efficacy that comes with surgical interventions, affecting not only the patient but health care systems and care-givers as well. Thus, it is important to concentrate efforts in identifying new therapeutic targets for OA. Achieving that goal may provide the necessary background that can lead us to the development of new specifically-target therapies.

Since disruption of autophagy has been suggested as a potential mechanism involved in OA, pharmacological activation of autophagy has been the subject of several studies. Different compounds are being studied as potential modulators of the autophagic pathway and consequently of OA progression (Carames et al, 2013; Li et al, 2014). Glucosamine is one of the compounds being studied as an autophagy modulator in chondrocytes. Both *in vitro* and *in vivo* experiments have shown that glucosamine is able to increase autophagy in chondrocytes and in articular cartilage and to protect nucleus pulposus cells through the inhibition of the mTOR pathway, pointing glucosamine as a potential therapeutic agent for autophagy modulation (Carames et al, 2013; Jiang et al, 2014). However, a time-dependent dual role of glucosamine in autophagy modulation in human chondrocytes was proposed - short time exposure increases autophagy while long time exposure inhibits autophagy (Kang et al, 2015b). Other drugs like dexamethasone also appear to present the same dual profile of action in autophagy (Liu et al, 2014). This dual effect can threaten their utility as a therapeutic option and needs to be clarified. It is essential to ascertain how long the drug remains at therapeutic concentrations in the site of action and if this type of exposure can lead to a short-term like effect or a long-term like effect. Whether this dual effect in chondrocytes is common to all autophagy inducers remains to be determined and is a question in need of further research.

Rapamycin is a well-known and widely used drug for autophagy activation through the inhibition of mTORC1 in many cell types. It has been used as an immunossupressive drug in transplantation and autoimmune disorders by inhibiting immune response; and in the treatment of cancer by inhibiting growth and proliferation of mammalian cells (Lamming et al, 2013). In articular cartilage, Rapamycin has also been tested, in both *in vivo* and *in vitro* models, to explore its possible protective role. In *in vitro* studies Rapamycin appeared to protect articular cartilage from oxidative stress, and cell death (Carames et al, 2012b; Lopez de Figueroa et al, 2015; Sasaki et al, 2012). Moreover, Rapamycin was also able to increase mRNA expression of aggrecan and type II collagen and decrease the expression of MMP-13 in OA chondrocytes (Zhang et al, 2015). *In vivo* studies, using C57Bl/6J mice with experimentally-induced OA, by transection of the medial meniscotibial

ligament and the medial collateral ligament, confirmed that the intraperitoneal administration of Rapamycin affects mTOR signaling, inducing autophagy, with increased LC3-II levels and decreased rpS6 phosphorylation in articular cartilage. Rapamycin treatment also decreased OA-like alterations, maintained cartilage cellularity and protected against ECM damage, by reducing ADAMTS-5 expression. Furthermore, Rapamycin also reduced the severity of synovitis and decreased the expression of IL-1β. Together, these results suggest that Rapamycin is able to reduce the severity of experimental OA, probably by modulating autophagy activation (Carames et al, 2012a). However, systemic long-term administration of Rapamycin has been associated with some side effects including edema, mucositis, hair and nail disorders, and dermatological effects, among others, making its utility problematic (Lamming et al, 2013). In order to avoid these side effects, some researchers suggest an intra-articular injection of the drug as a more appropriate route for clinical use. The intra-articular injection of Rapamycin was performed in mice with surgically-induced OA (DMM model). This administration was able to reduce articular degeneration and preserve hyaline cartilage, by downregulating cartilage degrading enzymes (MMP9 and MMP13), hypertrophy-related genes (vascular endothelial growth factor-VEGF- and COL10A1), inflammation-related genes (IL-1β and IL-6), and stress-responsive genes (CCAAT/enhancer binding protein beta- C\EBPβ and mTOR) and by upregulating Col2a1 (Matsuzaki et al, 2014; Takayama et al, 2014). These studies show that intra-articular administration of Rapamycin is able to maintain the protective effect, while potentially avoiding the side effects observed with systemic administration.

Despite the unequivocal effect of mTOR in autophagy regulation, it also induces a negative feedback loop in the PI3K/Akt pathway. mTOR activation increases p70S6K, that is responsible for the phosphorylation and consequent inhibition of the insulin receptor substrate (IRS1), one upstream regulator of the PI3K/Akt pathway. Oppositely, mTOR inhibition leads to an enhancement of the PI3K/Akt pathway (Wullschleger et al, 2006). This PI3K/Akt activation can then increase MMP production by chondrocytes. Based on this knowledge, Chen J. *et al*, proposes a dual inhibition of both mTOR and PI3K/Akt as a more promising approach for OA, abolishing the negative feedback mechanism and its possible side effects (Chen et al, 2013).

Another important point to consider is that excessive inhibition of mTOR expression could be harmful for tissues. According to Tchetina, E.V. *et al*, OA outpatients with lower mTOR gene expression in PBMCs compared to healthy controls, exhibited more pain while standing and upon joint function, and increased total joint stiffness than OA outpatients with higher mTOR gene expression (Tchetina et al, 2013). These harmful effects of persistent autophagy activation were also reported in other studies. For instance, chronic autophagy activation was detected in mouse models

of premature aging and this seems responsible for the systemic degeneration and weakening (Marino et al, 2008). Therefore, continuous autophagy activation is likely not the best therapeutic approach. Instead, repairing autophagic defect, or restoring the normal levels of autophagy, could be a more efficient approach for many diseases, including OA.

1.5 OBJECTIVES AND STRUCTURE OF THE THESIS

Notwithstanding the potential effects of other forms of diabetes, namely type 1, in articular cartilage and other joint tissues, this thesis mainly focus in T2D, given the growing societal importance of both T2D and OA, as detailed in the introduction, as well as increasing evidence indicating the existence of casual relationships between these diseases. Thus, the general objective of this thesis is to contribute to a better understanding of the mechanisms involved in such association.

In particular and given the importance of autophagy as a crucial homeostatic mechanism known to be dysregulated both in OA and in diabetes, but differentially modulated in distinct cells and tissues involved in diabetic complications, the major objective and focus of this thesis is to understand whether diabetic conditions directly modulate autophagy in chondrocytes and joint tissues and whether it contributes to cartilage and joint damage. Directly in line with this, a subsidiary objective is to determine if pharmacological autophagy activation can counteract the deleterious effects of diabetes in chondrocytes, articular cartilage, and the joint and thus, whether autophagy modulation can be a potential target for the development of novel more effective therapeutic approaches.

To achieve that, we used an *in vitro* model mimicking hyperglycemia and hyperinsulinemia, hallmarks of T2D, that are also present in type 1 diabetic patients under insulin therapy. Thus, human primary articular chondrocytes and articular cartilage explants and a chondrocytic cell line were cultured in a high glucose medium and exposed to high insulin concentrations. The expression of autophagy markers (LC3) and catabolic factors (MMP-13 and IL-1β) was evaluated to understand the effects of diabetes in chondrocyte autophagy and in cartilage integrity. Moreover, these responses were also evaluated after autophagy activation, to explore the possible beneficial effects of this modulation.

Then, to confirm the relevance of these findings under more complex dynamic conditions that more closely mimic the human disease, an *in vivo* study was performed using an animal model of T2D and non-diabetic lean mice, that were subjected to surgical OA induction. The db/db animal model is one of the best characterized and most commonly used animal models for investigation on T2D, and was chosen to set about the present study. Db/db mice are hyperphagic, obese, hyperglycemic, and hyperinsulinemic, thus representing a model of metabolically compromised animals. These metabolic disturbances are similar to those observed in T2D in humans, making it a good model to study the effects of diabetes in many tissues, including articular cartilage. The animal

models of OA can be divided into induced and spontaneous models and OA induction can be achieved surgically or chemically. Surgical models seem to represent more closely the histopathologic changes and clinical course of human OA than the chemical models (Lampropoulou-Adamidou et al, 2014). Therefore, to perform this study we chose a surgical model that involves the transection of the medial meniscotibial (MMTL) and the medial collateral (MCL) ligaments. The transection of these ligaments causes instability of the knee which is sufficient to induce the development of a slowly progressing form of OA, mimicking a condition equivalent to human OA that displays mild to moderate joint damage. To pharmacologically induce autophagy, Rapamycin, a well-known autophagy activator was used. Db/db mice were divided in two groups and treated with Rapamycin or vehicle. At the end of the study, histological analyses, as well as analysis of the expression of OA biomarkers in articular cartilage, were performed.

The results and discussion of the *in vitro* study are presented in chapter 2, while those of the *in vivo* study are presented in chapter 3.

The last chapter of this thesis presents a comprehensive review of the results, and tries to identify remaining gaps concerning the role of diabetes in OA and future perspectives for the development of innovative targeted therapies to tackle this new epidemic.

CHAPTER 2

INSULIN DECREASES AUTOPHAGY AND LEADS TO CARTILAGE DEGRADATION

Ribeiro M, López de Figueroa P, Blanco FJ, Mendes AF, Caramés B

Insulin decreases autophagy and leads to cartilage degradation

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ABSTRACT

Objective: Autophagy, a key homeostasis mechanism, is defective in Osteoarthritis (OA) and Type 2 Diabetes (T2D). T2D has been proposed as a risk factor for OA. We hypothesized that diabetes impairs articular cartilage integrity by decreasing autophagy. Our objective was to investigate the effects of high glucose and insulin, characteristics of T2D, on cartilage homeostasis.

Methods: Immortalized human chondrocytes (TC28a2) and primary human chondrocytes were cultured in 25 mM or 0 mM glucose and treated with insulin (10, 100, 500 nM) for 2, 6 or 24 h. Activity of LC3-II, Akt and rpS6 was evaluated by Western blotting (WB). Human cartilage explants were cultivated with 25 mM glucose and insulin (100,1000 nM) for 24 h to evaluate histopathology. MMP-13 and IL-1β expression was determined by immunohistochemistry and WB. Effects of Rapamycin (10 µM) were analyzed by WB. LC3 and rpS6 expression was determined by WB in chondrocytes from Healthy, Non Diabetic-OA and Diabetic-OA patients.

Results: Insulin downregulates autophagy by reducing LC3 II expression and increasing Akt and rpS6 phosphorylation. Loss of proteoglycans and increased MMP-13 and IL-1β expression was observed after insulin treatment. Autophagy activation by rapamycin reversed insulin effects. Importantly, chondrocytes from diabetic-OA patients showed decreased LC3 and increased p-rpS6 expression compared to Healthy and Non-Diabetic OA patients.

Conclusions: These results suggest that decreased autophagy might be a mechanism by which diabetes influences cartilage degradation. Pharmacological activation of autophagy may be an effective therapeutic approach to prevent T2D-induced cartilage damage.

Keywords: Insulin; Autophagy; Chondrocytes; Osteoarthritis (OA); Diabetes.

2.1 INTRODUCTION

Osteoarthritis (OA) and Type 2 diabetes (T2D) are age-related diseases becoming more prevalent due to increased human longevity (Guariguata et al, 2014; Martin & Buckwalter, 2002). Epidemiological studies suggest a possible association between OA and T2D, where diabetes could be an important risk factor for OA (Berenbaum, 2011; Schett et al, 2013; Sturmer et al, 2001). However, the molecular mechanisms underlying this correlation are not yet well understood.

OA, a degenerative joint disease that affects millions of people worldwide, is a major cause of disability and pain in older adults (Loeser et al, 2012). Among other risk factors for the development of OA, such as aging, genetic predisposition and mechanical forces, metabolic factors are critical for joint damage (Lee et al, 2013). Several lines of evidence suggest that glucose metabolism and hormonal regulation can influence cartilage integrity (Sellam & Berenbaum, 2013). Hyperinsulinemia and insulin resistance, the major features of T2D (Moller & Kaufman, 2005) are associated with the inability to transport and metabolize glucose into tissues (i.e., liver, muscle and adipose tissue). The abnormally elevated levels of circulating glucose force the pancreas to produce more insulin. In the pancreas, beta cells synthesize insulin, which is stored in vacuoles and released in response to high blood glucose levels. Prolonged metabolic demands eventually may cause beta cell dysfunction and diabetes, leading to severe damage in heart, blood vessels, eyes, kidneys and nerves (American Diabetes, 2013; Yan & Li, 2013). Hyperinsulinemia is most often caused by insulin resistance at the early stages of diabetes and may promote cartilage degradation. For example, advanced glycation end products, which are proteins or lipids that become glycated after exposure to sugars, are accumulated in aged cartilage (DeGroot et al, 2004). In addition, long-term insulin therapy often needed to treat diabetes may overload tissues such as cartilage. Indeed, joint damage severity is higher in diabetic patients (King et al, 2013; Martinez-Huedo et al, 2013). Whether high glucose and insulin levels can cause damage to articular cartilage and contribute to the development of OA remains to be clarified.

Autophagy is a cellular homeostasis mechanism responsible for maintenance of the metabolic balance, mainly through the removal and degradation of damaged intracellular products, which are sequestered in autophagosomes for degradation and utilization. Autophagy is regulated by many genes, including the microtubule-associated protein 1 light chain 3 (LC3), a major autophagy effector, and the mammalian target of rapamycin (mTOR), an autophagy repressor (Levine & Kroemer, 2008). During autophagy activation, LC3 I is converted to LC3 II through lipidation in the autophagosomes (Kabeya et al, 2000). Mammalian TORC-1 is regulated by signaling pathways involving PI3K/Akt and regulates protein synthesis by phosphorylating ribosomal S6 protein kinase

(rpS6) (Conn & Qian, 2011; Fenton & Gout, 2011). Autophagy has been implicated in pathogenesis of various human diseases such as cancer, neurodegenerative disorders and infectious diseases (Jiang & Mizushima, 2014; Sridhar et al, 2012). In aging and OA cartilage, autophagy is defective (Carames et al, 2010). In human OA cartilage, mTOR is overexpressed and the genetic loss-of-function increases autophagy signaling (Zhang et al, 2015), while pharmacological activation of autophagy by mTOR inhibition protects against cartilage degradation and reduces OA severity (Carames et al, 2012a; Sasaki et al, 2012). Autophagy is also relevant in metabolic diseases, including diabetes (Ryter et al, 2014). Defective autophagy is found in various cell types such as pancreatic beta cells, renal tubular cells and adipocytes (Masini et al, 2009; Ost et al, 2010). Moreover, pharmacological inhibition of mTOR has been proposed as a potential therapeutic strategy for obesity and T2D, since an imbalance in mTOR pathway is observed in these pathologies (Dann et al, 2007). However, how T2D may influence autophagy function and OA disease progression in human chondrocytes and cartilage is still unknown.

The objective of this study was to investigate the role of autophagy in articular cartilage under diabetic conditions. Human chondrocytes and cartilage were subjected to high glucose and insulin and its effects on the regulation of autophagy were studied.

2.2 METHODS

2.2.1 CULTURE OF CELL LINES IN MONOLAYER

Immortal juvenile human primary chondrocytes, TC28a2, obtained as described previously were employed in this study (Goldring et al, 1994). The cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and maintained at 37 ºC, in the presence of 5% CO_2.

2.2.2 CHONDROCYTE ISOLATION AND CULTURE

Normal human articular cartilage was harvested at the time of autopsy from the femoral condyles and the tibial plateaus of patients who had no history of joint disease (mean age ± SD 76 ± 12.63, n=6). OA human cartilage was obtained from patients undergoing knee replacement. Briefly, cartilage slices were incubated for 15 minutes at 37°C with trypsin (0.5 mg/ml; Sigma-Aldrich). After the trypsin was removed, the cartilage slices were incubated overnight at 37°C with 2 mg/ml of clostridial collagenase (Sigma-Aldrich) in DMEM with 5% FBS, with shaking. The isolated chondrocytes were recovered and plated in DMEM supplemented with 10% FBS, L-glutamine, and antibiotics and were allowed to attach to the culture flasks. The cells were incubated at 37°C in a humidified gas mixture containing 5% CO2 balanced with air. The chondrocytes were used in the experiments at confluence. The Ethics Committee of Galicia, Spain, approved this study. Chondrocytes from healthy patients (mean age ± SD 54 ± 1.41, Mankin score 1, mean Body mass index (BMI) ± SD 21.57 ± 1.77, n=2), non-diabetic-OA patients (mean age ± SD 78.75 ± 11.52, Mankin score 6, mean BMI ± SD 25.725 ± 3.42, n=4) and from diabetic-OA patients (mean age ± SD 70.75 ± 10.17, Mankin score 8, mean BMI ± SD 26.775 ± 8.55, n=4) diagnosed with OA and T2D were used.

2.2.3 CELL AND CARTILAGE CULTURE CONDITIONS

TC28a2 cells and human chondrocytes were plated in 8-well chamber slides (1 x 10^5 cells), or 6-well plates (5 x 10^5 cells) or 12-well (2.5 x 10^5 cells) plates, in high glucose DMEM 25mM (Lonza; BE12-604F) containing 2% FBS or in DMEM 0mM glucose (Gibco-Life Technologies; 11966-025) containing 2% FBS. Insulin (10, 100 or 500nM) (Sigma-Aldrich, St. Louis, MO, USA; I0516) or autophagy inducer Rapamycin (10µM, Calbiochem) were added and incubated for the indicated times. Rapamycin was applied as positive control of autophagy activation for LC3 quantification.

2.2.4 ARTICULAR CARTILAGE EXPLANTS

Full thickness normal human articular cartilage (mean age ± SD 68 ± 10.07, n=3) explants without subchondral bone were harvested from femoral condyles of human articular cartilage. Explants (6 mm in diameter) were performed and maintained in DMEM supplemented with 10% FBS. For experiments, explants were cultured overnight in high glucose DMEM containing 2% FBS and treated with 100 and 1000nM of insulin for 24 h.

2.2.5 WESTERN BLOTTING (WB)

Cell lysates were prepared using 6M urea/2%SDS buffer and a total 25µg protein of each cell lysate was separated on 4- 20% SDS-polyacrylamide gels. WB was performed as described previously (Lopez de Figueroa et al, 2015). The primary antibodies employed are presented on Table 2.1.The intensity of the bands was analyzed by using TotalLab (TL120) software.

Table 2. 1 - List of primary antibodies used in Western Blot of cellular extracts

Protein	Source	Clonality	MW (KDa)	Dilution	Supplier/ Ref. number
LC3	Rabbit	Polyclonal	16-18	1:1000	MBL International/ PM036
p-Akt (Ser 473)	Rabbit	Monoclonal	60	1:2000	Cell Signaling Tech., Inc./ 4060
p-rpS6 (Ser 235/236)	Rabbit	Monoclonal	32	1:2000	Cell Signaling Tech., Inc./4858
IL-1β	Rabbit	Polyclonal	31	1:500	Santa Cruz BioTech., Inc./sc-7884
α-Tubulin	Mouse	Monoclonal	50	1:2000	Sigma-Aldrich®/ T9026

2.2.6 IMMUNOCYTOCHEMISTRY

Chondrocytes were fixed with paraformaldehyde for 10 minutes, and permeabilized with 0.3% Triton X-100 in PBS for 15 minutes at Room Temperature (RT). Then, the cells were blocked for 30 minutes in 5% Normal Goat Serum (NGS) in PBS and incubated with LC3 antibody (1:500; PM036) in 1% NGS/PBS at RT for 1h. Next, a fluorescent dye-conjugated secondary antibody (Alexa Fluor 488-conjugated rabbit IgG; 1:200 Life Technologies; A-11034) was added and incubated at RT for 30 minutes. The nuclear staining was analyzed with Hoechst 33342 (1 µg/ml, Life Technologies). Finally, the slides were mounted with ProLong® Gold Antifade Reagent (Life Technologies), and observed by fluorescence microscopy (Olympus). Images were imported into Image J software (National Institute of Health, USA) for quantitative image analysis.

2.2.7 HISTOLOGICAL ANALYSIS AND IMMUNOHISTOCHEMISTRY

Paraffin-embedded cartilage explants were first deparaffinized in xylene and rehydrated in graded ethanol and water. Serial sections (4μm) were cut in a microtome. Then, sections were stained with Safranin O-fast green to examine the extracellular matrix changes.

Sections were treated with 3% hydrogen peroxide for 10 minutes, blocked with 5% serum for 30 minutes at RT and incubated with primary antibodies presented on Table 2.2, overnight at 4ºC. Then, sections were incubated with biotinylated goat anti-rabbit secondary antibody for 30 minutes at RT, and then incubated for 30 minutes using the Vectastain ABC-AP kit (Vector, Burlingame, CA). Slides were washed, and sections were incubated with 3,3-diamino-benzidine (DAB) substrate for 3–10 minutes.

LC3 and MMP13 expression was determined by counting positive cells a minimum of 3 times for each section. The number of positive cells was counted in each section (Taniguchi et al, 2009).

Table 2. 2 - List of primary antibodies used in Immunohistochemistry of cartilage explants

Protein	Source	Clonality	Dilution	Supplier/ Ref. number
LC3	Rabbit	Polyclonal	1:1000	MBL International/ PM036
MMP-13	Mouse	Monoclonal	1:50	Thermo Scientific/ MS-825

2.2.8 STATISTICAL ANALYSIS

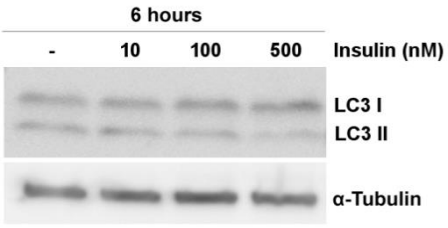
To test for normal distribution of the data, we used the Kolmogorov-Smirnov test. In general, the data sets follow a normal distribution. In the cases where the number of samples was too small to be tested accurately, we assumed the data follow a normal distribution. Statistically significant differences between 2 groups were determined by Student´s unpaired t-test. Statistically significant differences between multiple groups were determined analyzing variance (ANOVA) in conjunction with Tukey´s multiple comparison. Data are presented as the mean (lower level, upper level) (ll, ul) 95% confidence interval (CI). The data analysis and statistical interference was performed using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

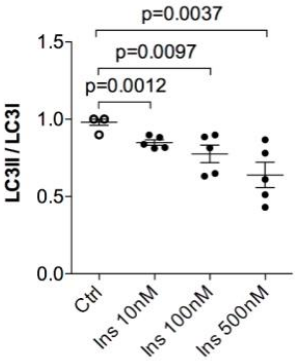
2.3 RESULTS

2.3.1 Reduced autophagy in response to high glucose and insulin in human chondrocytes and cartilage

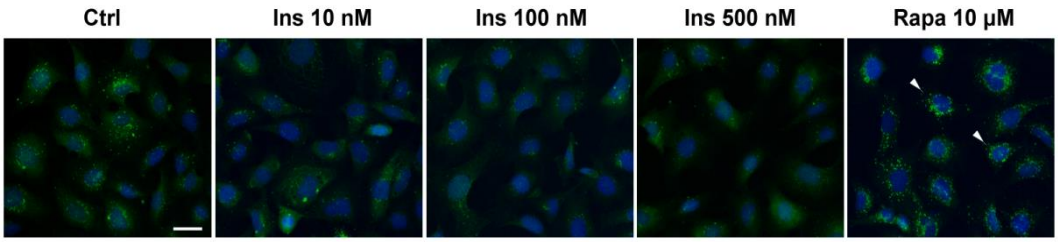
Autophagy is a central mechanism for cartilage homeostasis, although the effect of glucose and insulin in this recycling pathway remains unexplored. We analyzed the effect of high glucose and insulin on the autophagy pathway by determining LC3 I lipidation and conversion to LC3 II as a marker of autophagy activation and autophagosome formation (Mizushima et al, 2010). In response to insulin, the expression of LC3 II was significantly decreased at 6 h after treatment [Figure 2.1A] in human TC28a2 chondrocytes. Fluorescence-based detection of LC3 isoforms revealed that insulin treatment results in a significant reduction of LC3 puncta, indicative of reduced autophagosome formation [Figure 2.1B]. These results were consistent with the reduced activity of LC3 II observed in primary human chondrocytes and in human cartilage explants at 24 h after insulin treatment compared to control (Ctrl) [Figure 2.1C, D]. These data indicate a significant reduction of autophagy activity in response to high glucose and insulin in human chondrocytes.

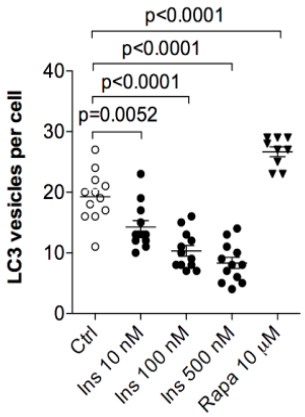
A


6 hours
-
10
100
500
Insulin (nM)
LC3 I
LC3 II
α-Tubulin


p=0.0037
p=0.0097
p=0.0012
LC3II / LC3I
1.5
1.0
0.5
0.0
Ctrl
Ins 10nM
Ins 100nM
Ins 500nM

B


Ctrl
Ins 10 nM
Ins 100 nM
Ins 500 nM
Rapa 10 µM


p<0.0001
p<0.0001
p<0.0001
p=0.0052
LC3 vesicles per cell
40
30
20
10
0
Ctrl
Ins 10 nM
Ins 100 nM
Ins 500 nM
Rapa 10 µM

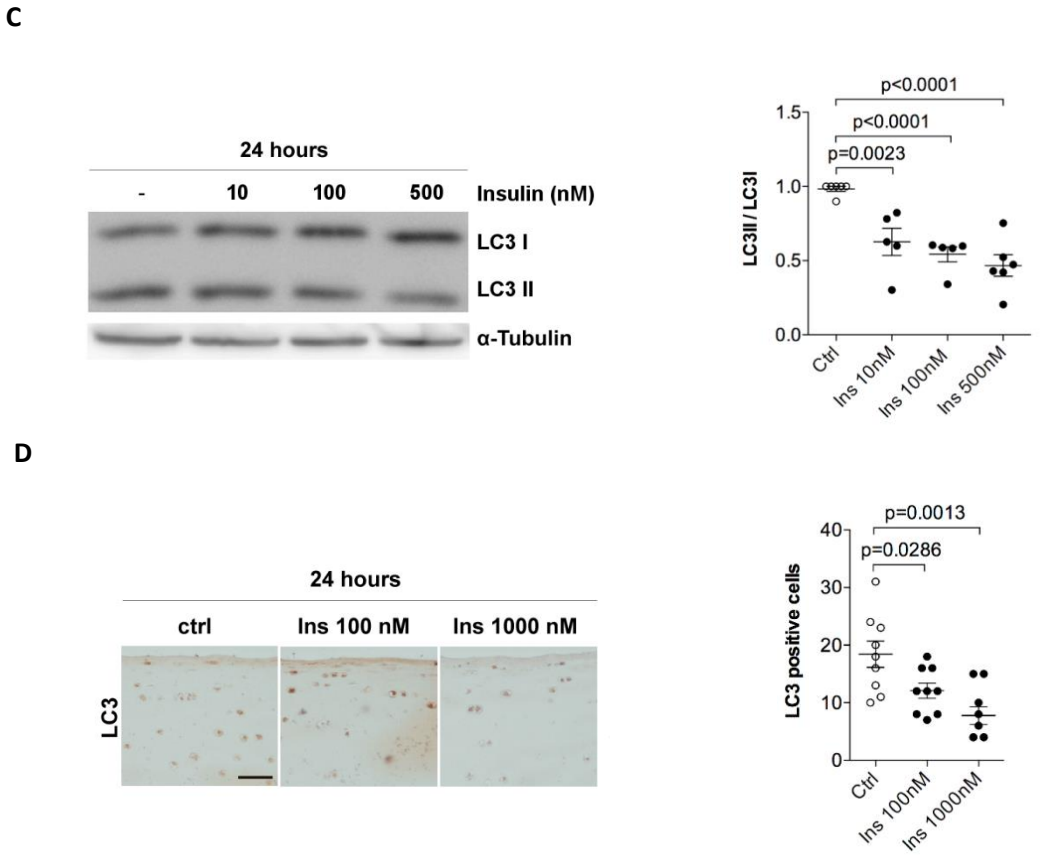


Figure 2. 1 - **Autophagy is reduced in response to high glucose and insulin in human chondrocytes and cartilage. A,** Western blot analysis to detect autophagosome formation by lipidation of LC3 I to LC3 II in human TC28a2 chondrocytes treated with Insulin (Ins; 10, 100, 500 nM) for 6 h. Values are the means (ll, ul) 95% CI of five repeated observations. **B,** Fluorescence-based detection of LC3 in TC28a2 cells treated with Insulin (10, 100, 500 nM) for 6 h. Scale bar 10 µm. Quantification of LC3 vesicles per cell. Values are the means (ll, ul) 95% CI of four repeated observations for triplicate (each point represents individual experimental repeat). Rapamycin (Rapa; 10 µM) was employed as a positive control for autophagy activation (LC3 vesicles: white arrows). **C,** Western blot analysis to detect lipidation of LC3 I to LC3 II in primary human chondrocytes treated with Insulin (10, 100, 500 nM) for 24 h. Values are the means (ll, ul) 95% CI of chondrocytes from five independent human donors (each point represents independent experimental values). **D,** Human cartilage explants were treated with Insulin (100, 1000 nM) for 24 h. Sections were analyzed by immunohistochemistry for LC3. Scale bar 100 µm. Values are means (ll, ul) 95% CI of two independent human cartilage donors for triplicate (each point represents independent experimental values).

2.3.2 DEPENDENCE OF HIGH GLUCOSE AND INSULIN-MEDIATED AUTOPHAGY DEFICIENCY ON AKT/mTOR PATHWAY

To understand the mechanism of action of high glucose and insulin on the autophagy pathway, we analyzed the phosphorylation of Akt, a regulator of insulin signaling, and rpS6, a direct and downstream target of mTOR, in human chondrocytes by WB. The results indicated a significant increase in p-Akt and p-rpS6 in response to insulin in a dose-dependent manner at 6 h in TC28a2 chondrocytes [Figure2. 2A]. These results were confirmed in primary human chondrocytes at 2 h after insulin stimulation [Figure 2.2B]. These data suggest that Akt/mTOR signaling pathway mediates the effect of high glucose and insulin on autophagy.

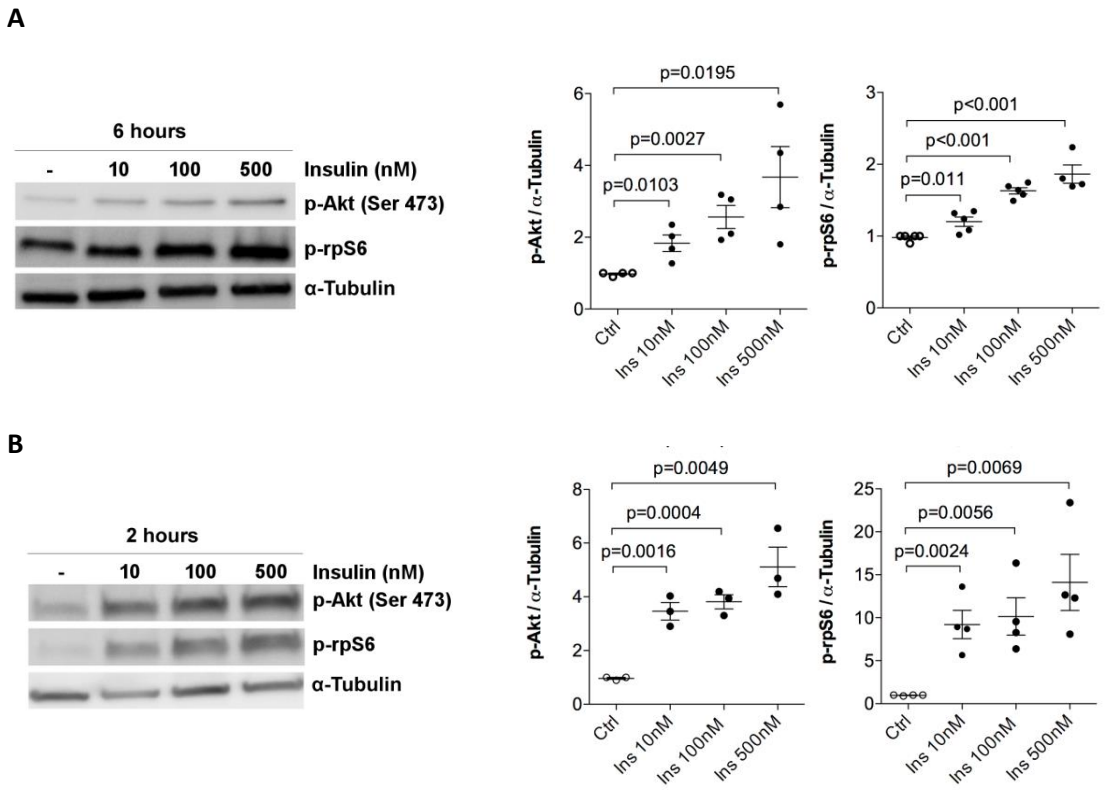


Figure 2. 2 - **High glucose and insulin-mediated autophagy reduction is dependent on Akt/mTOR pathway. A,** Human TC28a2 chondrocytes were untreated (DMEM HG 2% FBS) or treated with insulin (10, 100, 500 nM) for 6 h. Phosphorylation of Akt (Ser473) and ribosomal protein S6 (rpS6) was determined by WB. Values are the means (ll, ul) 95% CI of four repeated observations for p-Akt and five repeated observations for p-rpS6 (each point represents individual experimental repeats). **B,** Primary human chondrocytes were untreated (DMEM HG 2% FBS) or treated with Insulin (10, 100, 500 nM) for 2 h. Phosphorylation of Akt (Ser473) and ribosomal protein S6 (rpS6) was determined by WB. Values are the means (ll, ul) 95% CI of three independent human donors for p-Akt and four independent human donors for p-rpS6.

2.3.3 INSULIN MEDIATES AUTOPHAGY DEFICIENCY IN HUMAN CHONDROCYTES IN THE ABSENCE OF GLUCOSE

To determine whether the observed autophagy deficiency is due to high glucose and insulin or as a result of insulin treatment alone, we analyzed the LC3 I lipidation and conversion to LC3 II, and the phosphorylation of Akt and rpS6 in the absence of glucose, and with incremental concentrations of insulin (10, 100 and 500 nM) in human TC28a2 chondrocytes [Figure 2.3A]. In response to insulin treatment, the expression of LC3 II was significantly decreased at 6 h after treatment. This reduction was accompanied by increased phosphorylation of Akt and rpS6 [Figure 2.3B]. These results indicate that insulin alone is sufficient to mediates the reduction of autophagy in human chondrocytes.

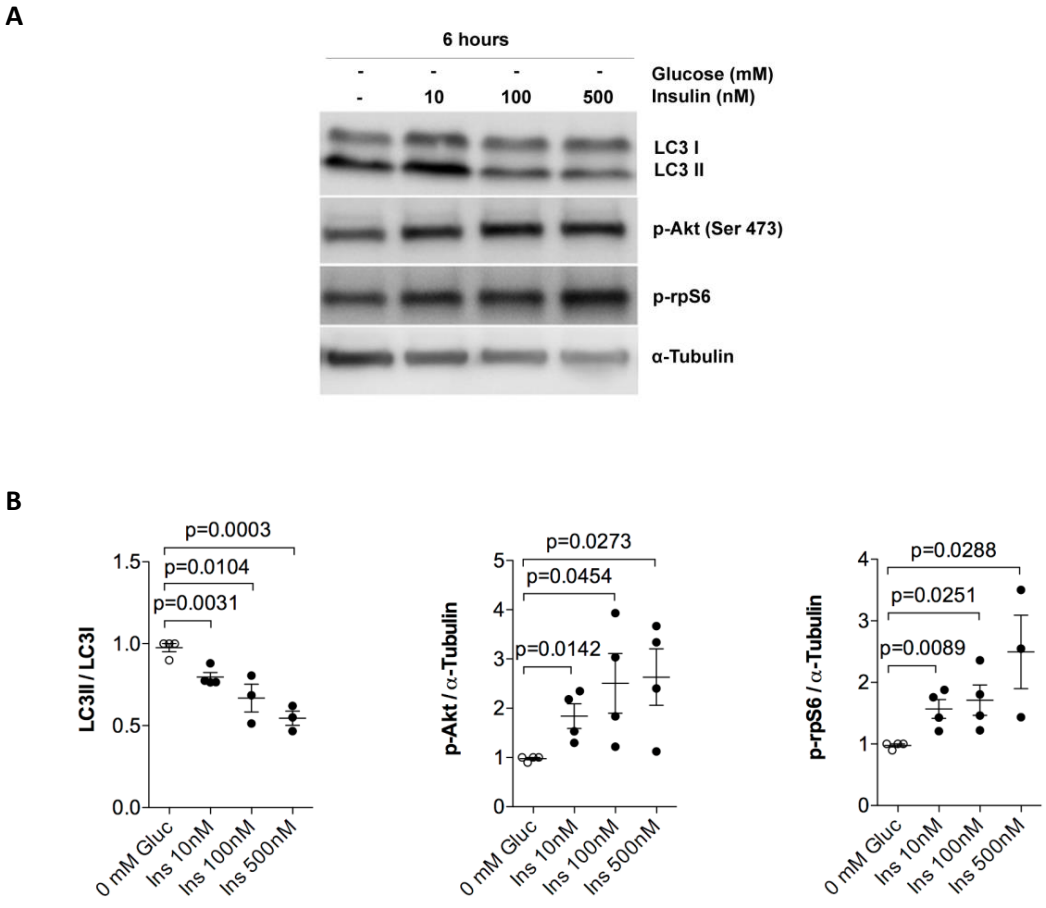


Figure 2. 3 - **Autophagy reduction in human chondrocytes is mediated by insulin. A,** Human TC28a2 chondrocytes were untreated (DMEM 2% FBS) in absence of glucose or treated with Insulin (10, 100, 500 nM) for 6 h. LC3 and phosphorylation of Akt (Ser473) and ribosomal protein S6 (rpS6) was determined by WB. **B,** Densitometric analysis of the ratio of LC3II/LC3I, p-Akt and p-rps6. α-Tubulin was employed as a loading control. Ratio of LC3II/LC3I: Values are the means (ll, ul) 95% CI of three repeated observations; p-Akt: Values are the means (ll, ul) 95% CI of four repeated observations; p-rps6: Values are the means (ll, ul) 95% CI of four repeated observations for Ctrl, Ins 10 nM, Ins 100 nM and three repeated observations for Ins 500 nM.

2.3.4 Loss of proteoglycans and upregulation of MMP-13 and IL-1β in human cartilage and chondrocytes by insulin

Diabetes is a risk factor for OA, but how diabetes regulates OA is still unknown (Schett et al, 2013). To determine the impact of insulin on cartilage integrity, human normal cartilage explants were stimulated with insulin for 24 h in the presence of high glucose. We studied IL-1β (a proinflammatory cytokine) and MMP-13 (a key metalloproteinase) because they are considered catabolic factors largely involved in cartilage degeneration and their production is mutually dependent (Fan et al, 2006). A loss of proteoglycans and a significant increase in MMP-13 expression was found after insulin treatment [Figure 2.4A]. Furthermore, increased IL-1β expression in TC28a2 chondrocytes is observed 6 h after insulin treatment [Figure 2.4B], indicating that insulin mediates inflammatory OA-like effects in articular cartilage and chondrocytes.

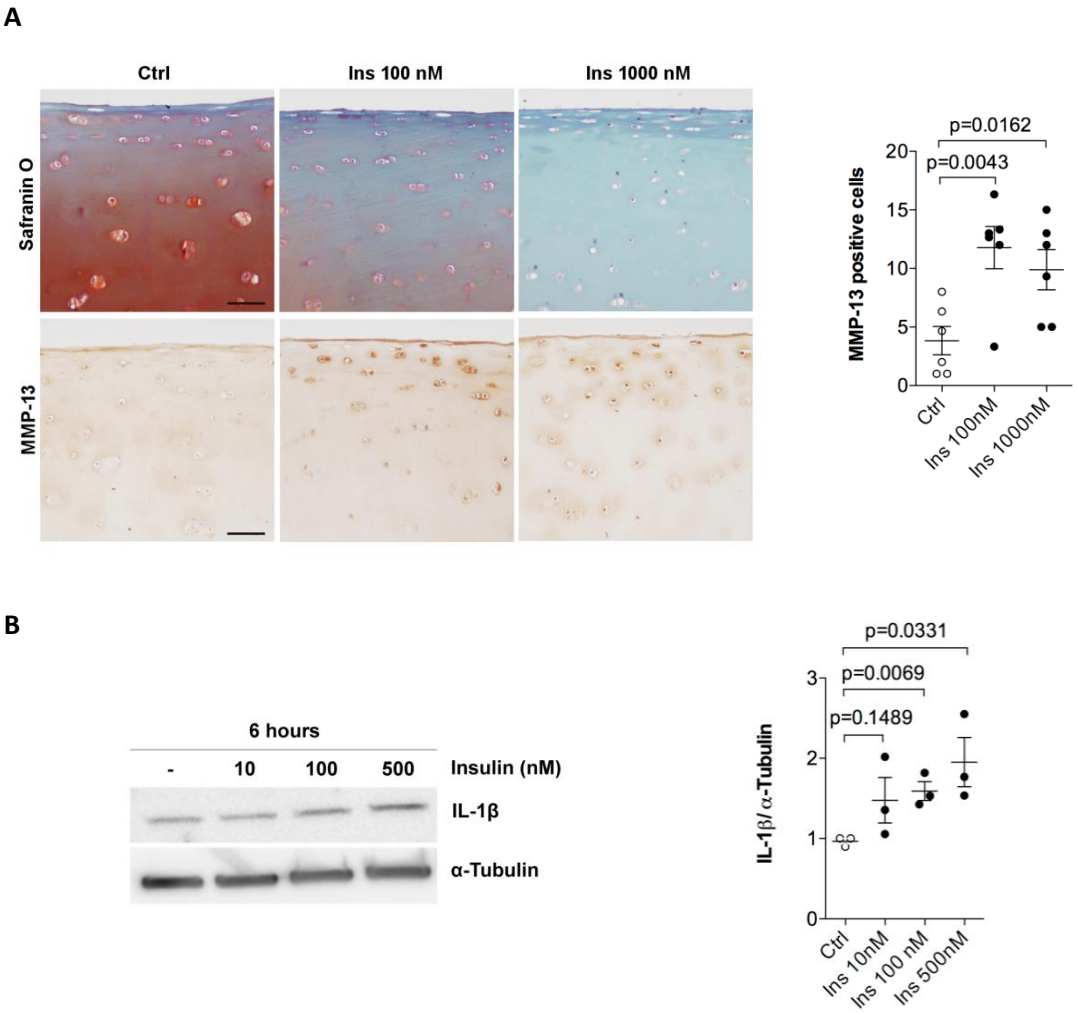
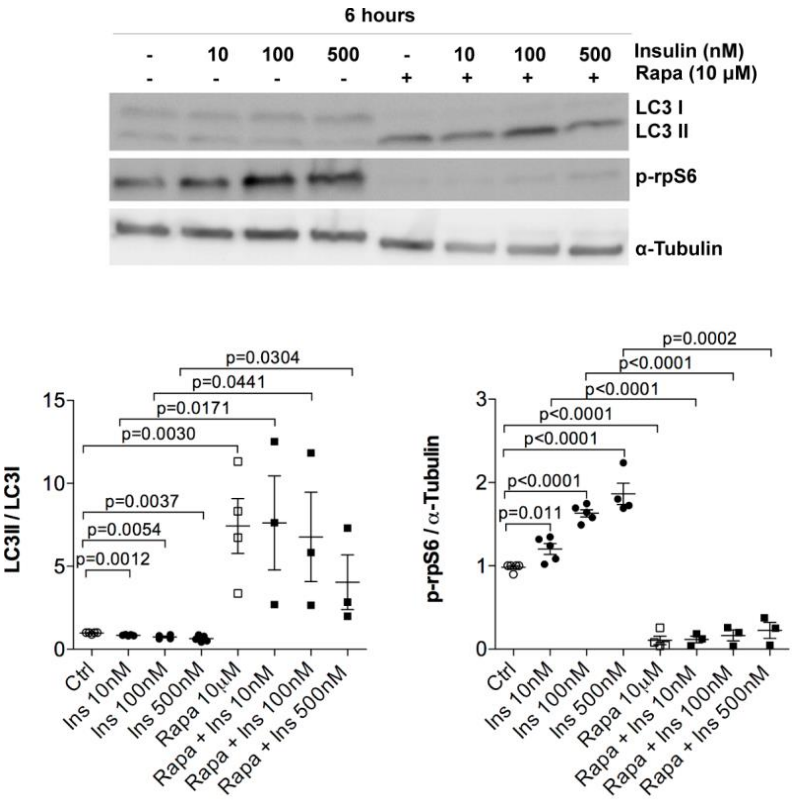


Figure 2. 4 - **Cartilage degradation and upregulation of OA biomarkers mediated by insulin. A,** Normal human cartilage explants were untreated (DMEM HG 2% FBS) or treated with Insulin (100, 1000 nM) for 24 h. Histology analysis was performed by Safranin O and MMP-13 expression was evaluated and quantified by immunohistochemistry. Values are the means (ll, ul) 95% CI of three independent human cartilage donors for duplicate. Magnification: 10x. Scale bar 100 µm. **B,** Human TC28a2 chondrocytes were untreated (DMEM HG 2% FBS) or treated with Insulin (10, 100, 500 nM) for 6 h. IL-1β expression was determined by WB. Values are the means (ll, ul) 95% CI of three repeated observations.

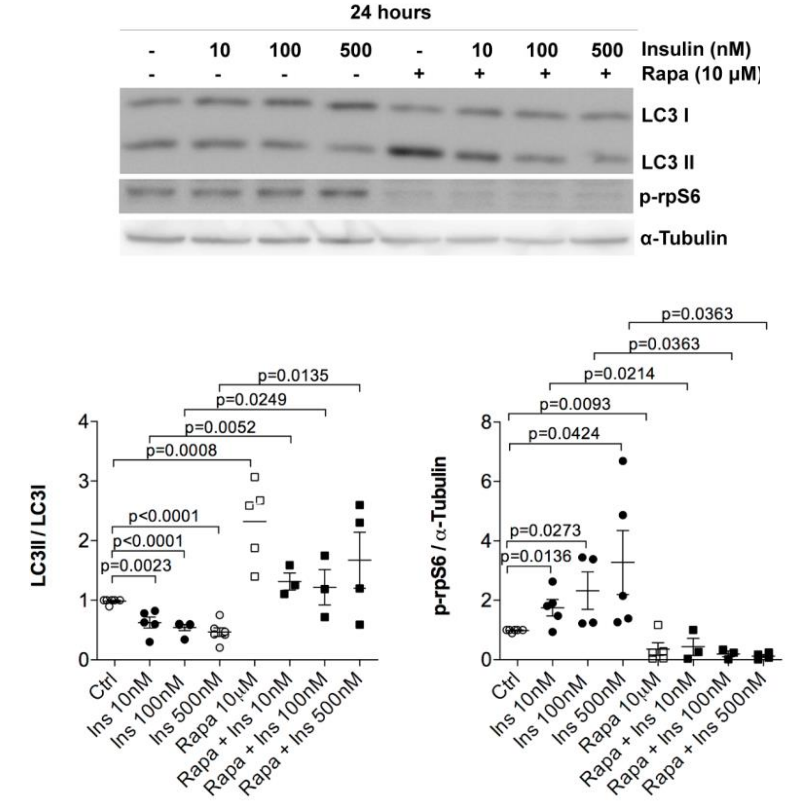
2.3.5 ACTIVATION OF AUTOPHAGY PROTECTS AGAINST INSULIN-MEDIATED AUTOPHAGY DEFECTS AND INFLAMMATION IN HUMAN CHONDROCYTES

To mTORC1 selective inhibitor rapamycin (10 µM) (Lamming et al, 2013; Lopez de Figueroa et al, 2015) was used to determine whether autophagy activation protects against insulin-mediated reduced autophagy. The results indicated that rapamycin reversed the deleterious effect of insulin on autophagy by maintaining the LC3 II expression in both TC28a2 chondrocytes and in primary human chondrocytes at 6 and 24 h, respectively. Rapamycin induction of autophagy was accompanied by reduced rpS6 phosphorylation [Figure 2.5A, B]. Interestingly, pharmacological autophagy activation by rapamycin significantly reduced the IL-1β expression promoted by insulin [Figure 2.5C], suggesting that autophagy activation protects against insulin-mediated autophagy reduction and the associated catabolic effects in articular cartilage.

A


6 hours
- 10 100 500 - 10 100 500 Insulin (nM)
- - - - + + + + Rapa (10 μM)
LC3 I
LC3 II
p-rpS6
α-Tubulin
LC3II / LC3I
p=0.0304
p=0.0441
p=0.0171
p=0.0030
p=0.0037
p=0.0054
p=0.0012
p-rpS6 / α-Tubulin
p=0.0002
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p=0.011
Ctrl
Ins 10nM
Ins 100nM
Ins 500nM
Rapa 10μM
Rapa + Ins 10nM
Rapa + Ins 100nM
Rapa + Ins 500nM

B


24 hours
- 10 100 500 - 10 100 500 Insulin (nM)
- - - - + + + + Rapa (10 μM)
LC3 I
LC3 II
p-rpS6
α-Tubulin
LC3II / LC3I
p=0.0135
p=0.0249
p=0.0052
p=0.0008
p<0.0001
p<0.0001
p=0.0023
p-rpS6 / α-Tubulin
p=0.0363
p=0.0363
p=0.0214
p=0.0093
p=0.0424
p=0.0273
p=0.0136
Ctrl
Ins 10nM
Ins 100nM
Ins 500nM
Rapa 10μM
Rapa + Ins 10nM
Rapa + Ins 100nM
Rapa + Ins 500nM

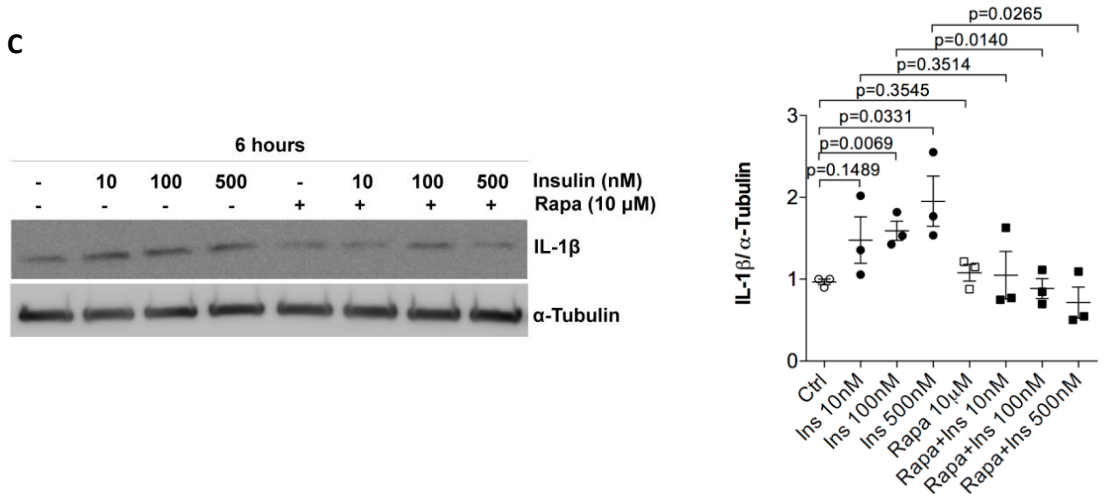


Figure 2. 5 - **Autophagy activation protects against insulin-mediated decreased autophagy and inflammation. A,** TC28a2 chondrocytes were treated with Insulin (10, 100, 500 nM) or Rapamycin (Rapa; 10 µM) for 6 h. LC3 and phosphorylation of ribosomal protein S6 (p-rpS6) were determined by WB. Ratio of LC3II/LC3I: Values are the means (ll, ul) 95% CI of five repeated observations for Ctrl and Ins 10-500 nM, four repeated observations for Rapa, and three repeated observations for Rapa + Ins 10, 100 and 500 nM. p-rpS6: Values are the means (ll, ul) 95% CI of five repeated observations for Ctrl, Ins 10-100 nM, four repeated observation for Ins 500 nM and Rapa, and three repeated observations for Rapa + Ins 10, 100 and 500 nM. **B,** Primary human chondrocytes were treated with Insulin (10, 100, 500 nM) or Rapa (10 µM) for 24 h. LC3 and p-rpS6 were determined by WB. Ratio of LC3II/LC3I: Values are the means (ll, ul) 95% CI of five repeated observations for Ctrl, Ins 10-500 nM and Rapa, three repeated observations for Rapa + Ins 10-100 nM, and four repeated observations for Rapa + Ins500 nM; p-rpS6: Values are the means (ll, ul) 95% CI of five repeated observations for Ctrl, Ins 10, 500 nM, and Rapa, four repeated observations for Ins 100 nM and Rapa + Ins500 nM, and three repeated observations for Rapa + Ins 10 and100 nM. **C,** TC28a2 chondrocytes were treated with Insulin (10,100,500 nM) or Rapa (10 µM) for 6 h. IL-1β expression was determined by WB. Values are the means (ll, ul) 95% CI of three repeated observations of each condition.

2.3.6 REDUCED AUTOPHAGY IN HUMAN CHONDROCYTES OF OA DIABETIC PATIENTS

Several studies support that OA is more prevalent and severe in T2D patients (King et al, 2013; Martinez-Huedo et al, 2013; Sturmer et al, 2001). We examined basal autophagy and mTOR signaling pathway in human chondrocytes from healthy, non diabetic-OA and diabetic-OA human cartilage [Figure 2.6A]. LC3 II expression was reduced in non diabetic-OA and diabetic-OA patients compared to healthy patients respectively. Importantly, LC3 II expression was significantly decreased in diabetic-OA patients compared to non diabetic-OA patients [Figure 2.6B]. This reduction in autophagy was accompanied with a significant increase in p-rpS6 expression in diabetic-OA patients compared to healthy and non diabetic-OA patients respectively [Figure 2.6B]. These results suggest that autophagy reduction might contribute to insulin-induced cartilage degradation in OA.

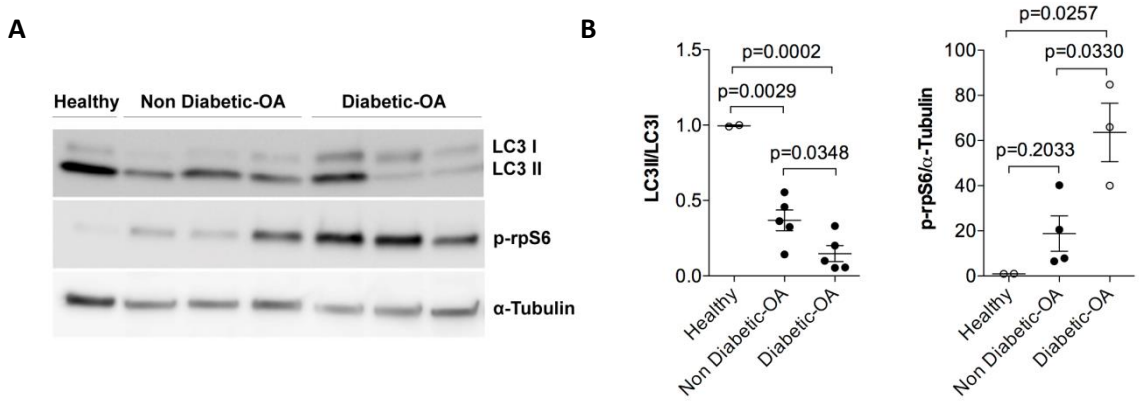


Figure 2. 6 **- Autophagy is reduced in human chondrocytes of OA diabetic patients. A,** Primary human chondrocytes from healthy, non diabetes-OA and diabetic-OA human cartilage were employed. LC3 and phosphorylation of ribosomal protein S6 (rpS6) were determined by WB. **B,** Densitometry analysis of the ratio of LC3II/LC3I expression and phosphorylation of ribosomal protein S6 (prpS6) expression. Ratio of LC3II/LC3I: Values are the means (ll, ul) 95% CI of four independent human donors; p-rpS6: Values are the means (ll, ul) 95% CI of four independent human donors for Non-Diabetic-OA chondrocytes and three independent human donors for Diabetic-OA chondrocytes.

2.4 DISCUSSION

We investigated the mechanisms by which T2D induces cartilage degradation. A direct association between OA severity and T2D has been proposed previously. Diabetic patients have a greater risk for developing OA than non-diabetic patients (King et al, 2013; Martinez-Huedo et al, 2013; Sturmer et al, 2001). However, the mechanism underlying this association and how diabetes accelerates cartilage degradation remains unknown. According to previous studies, high glucose concentrations favor catabolism in human chondrocytes and facilitate the development and progression of OA (Rosa et al, 2011b). Nevertheless, the effects of both high glucose and insulin, the hallmarks of T2D and insulin resistance, have not been investigated in cartilage.

Articular cartilage is a tissue characterized by a low cell turnover (Goldring & Goldring, 2007) and we hypothesize that autophagy may be one of the pathways regulating the effects of hyperglycemia and hyperinsulinemia in joint integrity. Indeed, autophagy plays an important role in articular cartilage, regulating the removal of dysfunctional organelles and macromolecules (Lotz & Carames, 2011). Basal levels of autophagy activity are indispensable for the homeostasis of many tissues, including cartilage. In opposite, defects in basal levels of autophagy have been associated with various degenerative and metabolic diseases (Sridhar et al, 2012). Previous studies reported a dysfunction of autophagy, either in OA and T2D. Defective autophagy and upregulated mTOR signaling in OA cartilage can be pharmacologically modulated to prevent the development and severity of OA (Carames et al, 2012a; Carames et al, 2010; Zhang et al, 2015). In diabetes, a dysfunction of autophagy was also described in metabolic tissues such as pancreatic beta cells and adipocytes. However, autophagy regulation in diabetic conditions is tissue-specific. Thus, nucleus pulposus cells from diabetic rats and diabetic adipocytes from human T2D patients show an increment in autophagy (Jiang et al, 2013; Ost et al, 2010). On the other hand, diabetic kidneys and hearts from diabetic animals exhibit a decrease on autophagy (Ding & Choi, 2015; Kobayashi & Liang, 2015).

These observations motivated us to investigate the role of autophagy in human chondrocytes and cartilage under high glucose and high insulin. First, human TC28a2 chondrocytes and primary human chondrocytes were maintained in high glucose medium and treated with increased doses of insulin (10, 100, 500 nM) in order to mimic the hallmarks of early stages of T2D. The insulin doses employed in this study were chosen according to previous studies (Montagnani et al, 2002; Rosa et al, 2011a; Sampson et al, 2010; Zeng et al, 2000). Autophagy was assessed by analysis of LC3, the key autophagy marker. Our findings demonstrated decreases in autophagy under high glucose and high insulin conditions in human chondrocytes. To elucidate the mechanism by which autophagy is

diminished, mTOR was evaluated. Our results indicated that insulin increased the mTOR signaling pathway in articular chondrocytes, as reflected by upregulated phosphorylation of ribosomal protein S6, a downstream target of mTOR responsible for regulating cell growth (Fenton & Gout, 2011). Moreover, we examined the phosphorylation of Akt, a major regulator of insulin signaling pathway (Mackenzie & Elliott, 2014). Our findings showed that insulin increased the Akt phosphorylation in a dose-dependent manner, suggesting that insulin effects are mainly dependent on the Akt/mTOR signaling pathway.

Since pharmacological activation of autophagy by mTOR inhibition exerts protective effects in cartilage (Carames et al, 2012b; Zhang et al, 2015), we investigated if rapamycin treatment can reverse insulin-mediated catabolic effects in chondrocytes. The results showed that rapamycin reversed the effects of insulin on autophagy activity in both TC28a2 and primary chondrocytes, as reflected by increased expression of LC3 II and decreased phosphorylation of rpS6. We anticipated that upregulating autophagy might have beneficial effects on cartilage integrity.

To evaluate the impact of insulin-mediated autophagy regulation in the context of cartilage biology, cartilage explants were treated with insulin (100, 1000 nM) for 24 h. Our results demonstrate that insulin decreased proteoglycan content and increased the expression of matrix metalloproteinase-13 (MMP- 13), which has a significant role in cartilage degradation and OA progression since preferentially cleaves type II collagen fibers (Takaishi et al, 2008). These data suggest that high insulin levels have catabolic effects in chondrocytes, without affecting cell viability (data not shown). In contrast to our observations, it has been reported that insulin can stimulate proteoglycan synthesis and reduce interleukin 1 (IL-1) effects, demonstrating an anabolic effect (Cai et al, 2002). The differences between both studies can be attributed to the insulin concentrations and the formulation employed. Our last piece of evidence demonstrate that autophagy activation by rapamycin was able to decrease the expression of IL-1β induced by insulin, indicating that inflammatory features in joints caused by high insulin levels might be prevented by regulating autophagy. To evaluate the insulin effect on IL-1 beta secretion, supernatants from cultured TC28a2 chondrocytes were used. However, the sensibility of the ELISA test was not enough to detect significant differences.

To investigate the role of autophagy in the progression of OA in diabetic patients, human chondrocytes were obtained from a small cohort of Non Diabetic-OA patients and Diabetic-OA patients. Basal autophagy by LC3 II expression and phosphorylation of rpS6 were evaluated. The results indicated a reduction in LC3 II expression, accompanied by an increased phosphorylation of rpS6 in Diabetic-OA patients, suggesting that decreased autophagy might be one of the mechanisms

responsible for the induced cartilage degradation observed in diabetic patients. Although the sample size of diabetic-OA patients is small, our results can be applicable as they shed light to patient-specific cartilage pathology.

Our study addresses the effect of diabetes on human chondrocytes and cartilage. All the results of our study were consistent in both T/C28a2 and primary chondrocytes. T/C28a2 immortalized human chondrocyte cell line express cartilage-specific genes such as SOX9, critical for chondrocyte differentiation, as well as type II collagen, which constitute 50% of all protein in cartilage. Although largely used as a model of chondrocyte function, it is proliferative and may not reflect the exact physiological conditions of primary chondrocytes. Studies on human chondrocytes and cartilage are critical to understand OA pathology.

In conclusion, this study demonstrates for the first time, that human chondrocytes and cartilage exposed to high glucose and high insulin have autophagy defects dependent on Akt/mTOR signaling pathway. We propose that pharmacological activation of autophagy may be an effective therapeutic approach for T2D-induced cartilage damage. Together, our data suggest that decreased autophagy may be one of the mechanisms responsible for the accelerated cartilage degradation observed in diabetic patients.

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CHAPTER 3

DIABETES- ACCELERATED EXPERIMENTAL OSTEOARTHRITIS IS PREVENTED BY AUTOPHAGY ACTIVATION

Ribeiro M, López de Figueroa P, Nogueira-Recalde U, Centeno A, Mendes AF,

Blanco FJ, Caramés B

Diabetes- accelerated experimental Osteoarthritis is prevented by autophagy activation

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ABSTRACT

Objective: Type 2 Diabetes (T2D) is a risk factor for Osteoarthritis (OA). Autophagy, an essential homeostasis mechanism in articular cartilage, is defective in T2D and OA. However, how T2D may influence OA progression is still unknown. We aimed to determine how diabetes affects cartilage integrity and whether pharmacological activation of autophagy has efficacy in diabetic mice (db/db mice) with OA.

Design: Experimental OA was performed in the right knee of 9 weeks-old C57Bl/6J male mice (Lean group, N=8) and of 9 weeks-old B6.BKS (D)-*Leprdb* male mice (db/db group, N=16) by transection of medial meniscotibial and medial collateral ligaments. Left knee was employed as control knee. Rapamycin (2 mg/kg weight/day) or Vehicle (dimethyl sulphoxide) were administered intraperitoneally 3 times a week for 10 weeks. Histopathology of articular cartilage and synovium was evaluated by using semiquantitative scoring and synovitis grading systems, respectively. Immunohistochemistry was employed to evaluate the effect of diabetes and Rapamycin on cartilage integrity and OA biomarkers.

Results: Cartilage damage was increased in db/db mice compared to lean mice after experimental OA, while no differences are observed in the control knee. Cartilage damage and synovium inflammation were reduced by Rapamycin treatment of OA-db/db mice. This protection was accompanied with a decrease in MMP-13 expression and decreased IL-12 levels. Furthermore, autophagy was increased and cartilage cellularity was maintained, suggesting that mTOR targeting prevents joint physical harm.

Conclusion: Our findings indicate that diabetic mice exhibit increased joint damage after experimental OA, and that autophagy activation might be an effective therapy for diabetes-accelerated OA.

3.1 INTRODUCTION

Osteoarthritis (OA) is the most common joint disease and represents an enormous socioeconomic burden affecting millions of people worldwide (Litwic et al, 2013).

Type 2 Diabetes (T2D) is a multifactorial chronic metabolic disease that results from defects in insulin secretion and/or action, resulting in hyperglycemia, hyperinsulinemia, relative β-cell dysfunction and insulin resistance, which is characterized by the failure of tissues to respond appropriately to insulin (American Diabetes, 2013).

As age-related diseases, both OA and T2D are expected to rise due to increase of life expectancy (Guariguata et al, 2014).

In OA, cartilage and many of its surrounding tissues are disrupted. Patients often experience pain, stiffness and loss of mobility, which leads to a functional impairment, incapacity and disability in elderly people (Glyn-Jones et al, 2015). With the increase of medical and disability costs and in the absence of an effective treatment for preventing OA, a better delineation of the risk factors becomes essential to investigate the cellular signaling pathways underlying the disease. This understanding will provide opportunities to identify new therapeutic targets that might predict OA severity and prevent progression.

Epidemiological and experimental reports suggest a positive association between OA and T2D, however, there is no comprehensive explanation for this association and further studies are required to understand the mechanism by which T2D accelerates OA progression (King & Rosenthal, 2015). T2D could be an important risk factor for OA (Berenbaum, 2011; Sturmer et al, 2001) by affecting cartilage homeostasis, accelerating OA progression and altering the prognosis by increasing the risk of total joint replacement (King et al, 2013; Martinez-Huedo et al, 2013; Schett et al, 2013). Thus, T2D patients seem to have an increased susceptibility to develop OA, which is independent of age, sex and body weight (Schett et al, 2013).

Our previous studies demonstrated that human chondrocytes from diabetic OA patients showed reduced autophagy, an essential cartilage homeostasis mechanism, which is associated to increased mammalian target of rapamycin (mTOR) activity compared to chondrocytes from non-diabetic OA patients. These results suggest that defective autophagy might be one of the mechanisms responsible for the cartilage degradation observed in diabetic patients (Ribeiro et al, 2016).

Autophagy is an intracellular mechanism that contributes to maintain cellular integrity, function and survival by the clearance of unnecessary/damaged proteins and organelles (Abounit et al, 2012). This mechanism is very important in poorly irrigated post-mitotic tissues, such as articular cartilage, with a very low rate of cell turnover and little regenerative capacity (Goldring & Goldring, 2007). Autophagy dysfunction contributes to the pathogenesis of many human diseases, including T2D and OA (Levine & Kroemer, 2008). Non-autonomous regulation of autophagy is observed in metabolic tissues relevant in T2D such as pancreas and adipose tissue (He et al, 2013; Inoki et al, 2011; Jung et al, 2008; Ost et al, 2010; Tagawa et al, 2016). In chondrocytes from OA patients, we demonstrated that autophagy markers are decreased, suggesting that compromised autophagy capacity could contribute to the development of OA (Carames et al, 2010).

Autophagy is regulated by multiple signaling pathways, most of them converging on mTOR. mTOR is a serine/threonine kinase that regulates cell growth, protein synthesis and metabolism, and it is considered a major negative regulator of autophagy (Wullschleger et al, 2006). mTOR plays an important role in age-related diseases including T2D and OA. Chronic activation of mTOR by growth factors and amino acids cause insulin resistance (Tremblay et al, 2005; Tremblay & Marette, 2001), indicating that dysregulation of mTOR pathway is intimately associated with diabetes (Dann et al, 2007). mTOR plays an important role in cartilage homeostasis, contributing to the degenerative process involved in OA. In fact, mTOR is overexpressed in human OA cartilage (Zhang et al, 2015), while mTOR inhibition prevents OA progression. In experimental OA performed in mice, both genetic deletion of mTOR and pharmacological inhibition of mTOR by Rapamycin lead to reduced OA severity (Carames et al, 2012a; Matsuzaki et al, 2014; Takayama et al, 2014; Zhang et al, 2015).

In the recent years the interaction between OA and T2D has been increasingly studied. However, lack of *in vivo* studies hampered the definition of mechanisms involved in the consequences of metabolic disease for OA. Thus, the objective of this study was to determine how diabetes affects cartilage integrity and to evaluate the efficacy of pharmacological activation of autophagy to reduce experimental OA in diabetic mice.

3.2 METHOD

3.2.1 ANIMAL STUDIES

Nine-week-old male *db/db* mice (B6.BKS (D)-*Leprdb*/J; JAX stock 000697) and the respective background strain mice (C57BL/6J-non-diabetic lean mice; JAX stock 000664) were purchased from The Jackson Laboratory. Db/db mouse, originated from C57BL/6J is one of the best characterized Type 2 diabetes (T2D) animal model. The db/db mouse lacks the leptin receptor due to a spontaneous autosomal recessive mutation. This mouse becomes obese around 3 to 4 weeks of age, with elevations of plasma insulin beginning at 10 to 14 days and of blood sugar at 4 to 8 weeks. Mice were housed in a 12h light/dark cycle and temperature-controlled environment, with ad libtium access to food and water. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Biomedical Research Institute (INIBIC) and in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

3.2.2 EXPERIMENTAL GROUPS AND RANDOMIZATION

Lean mice (C57BL/6J-Non-diabetic mice;N=8); db/db-Vehicle mice (N=8) and db/db-Rapamycin mice (N=8). Db/db mice were randomized according to body weight and blood glucose levels in two groups of N=8. At the beginning of the study the differences of body weight and blood glucose levels were not significantly different between both groups.

The schematic representation of the study design is presented in Figure 3.1.

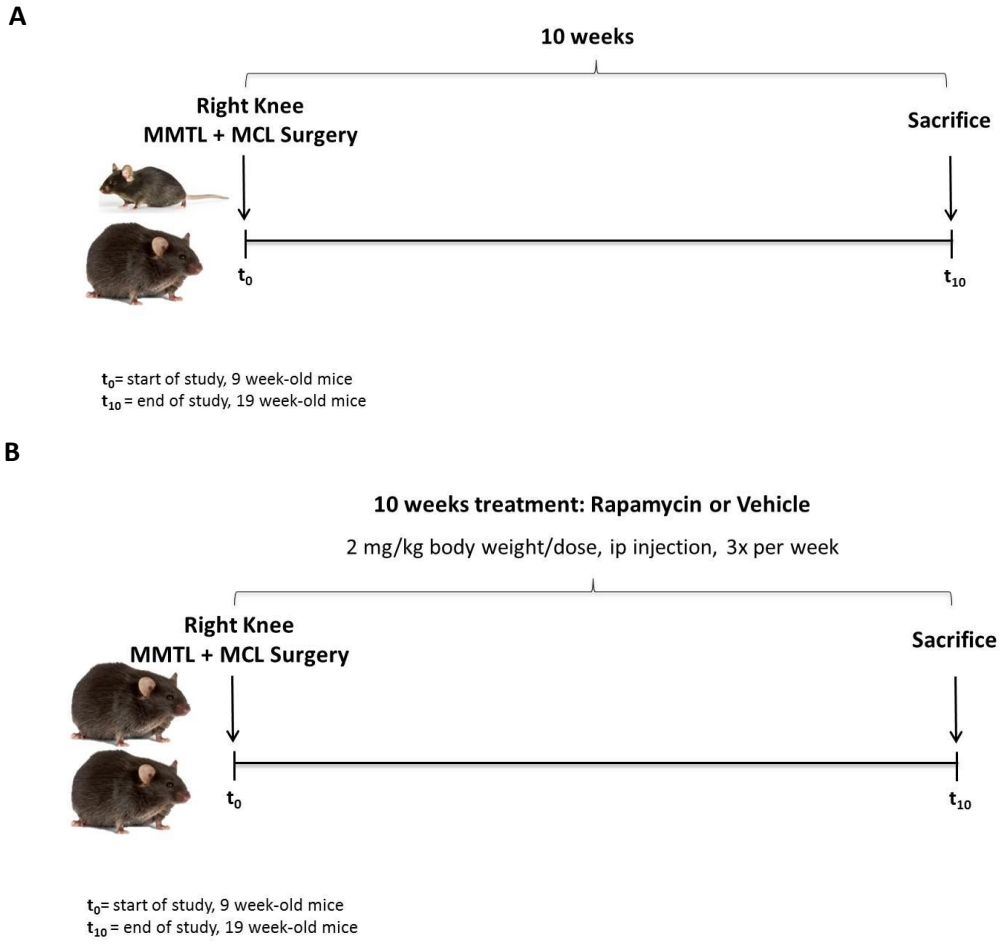

Figure 3. 1 **- Schematic representation of the study design.** OA was surgically-induced in all mice (9 week-old mice). 10 weeks after OA induction animals were sacrificed and histological analysis were performed. **A,** Lean mice were compared with db/db-vehicle mice. **B,** Db/db-vehicle mice were compared with db/db-Rapamycin mice. Starting one day after OA induction, Rapamycin (2mg/kg body weight/ dose) or Vehicle were administered intraperitoneally three days per week, during 10weeks.

3.2.3 EXPERIMENTAL OSTEOARTHRITIS

Following acclimation for one week, all animals were anesthesized with sevofurane and experimental OA was induced in nine weeks-old male mice by transection of the medial meniscotibial ligament and the medial collateral ligament (MMTL+MCL) in the right knee by a trained veterinary surgeon. The left knee was not subjected to surgery and was used as a control (Carames et al, 2012a). All mice were allowed to move freely within their cages after surgery. Recovery was monitorized and a clinical follow-up was performed in the following days. 10 weeks after the knee

surgery, the animals were fasted for 16 hours and then euthanized by CO_2 inhalation. A diagram of the mouse knee joint is presented in Figure 3.2.

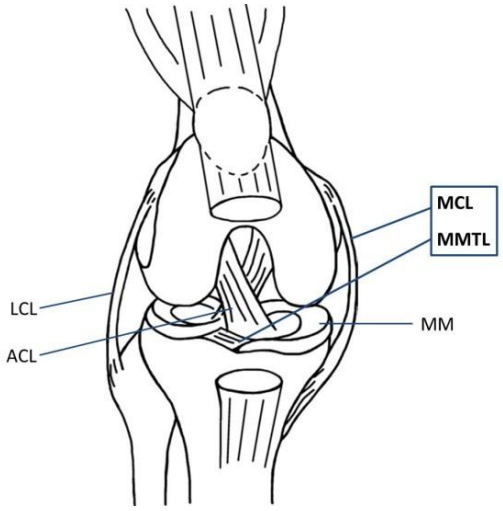

Figure 3. 2 - **Diagram of the mouse knee joint.** To surgically induce OA, the MMTL and MCL of the right knee were transected. MCL, medial collateral ligament; MMTL, medial meniscotibial ligament; MM, medial meniscus; ACL, anterior cruciate ligament; LCL, lateral collateral ligament. Adapted from (Kamekura et al, 2005)

3.2.4 RAPAMYCIN TREATMENT

Rapamycin purchased from LC Laboratories (Woburn, MA), was dissolved in dimethyl sufoxide (DMSO) at 25 mg/ml and stored at -20ºC. The stock solution was diluted in phosphate buffered saline (PBS) for injection. Treatment was started the day after the surgery and continued for 10 weeks. Rapamycin was injected intraperitoneally, three days per week at 2 mg/kg body weight/dose in a total injection volume of 0.2ml for 10 weeks. Importantly, the drug administration was well tolerated by all animals. The control group received the DMSO vehicle in PBS. The body weight was monitored weekly to adjust the dose of Rapamycin/Vehicle to inject. The dosage and frequency of Rapamycin administration were selected based on previous studies (Carames et al, 2012a; Mori et al, 2009).

The experimental groups employed in this study are summarized in Table 3.1.

Table 3. 1 - Experimental groups employed in this study

Group A: Lean	**Group B: Db/db-Vehicle**	**Group C: Db/db-Rapamycin**
C57BL/6J-Non-diabetic mice, Lean mice	B6.BKS (D)-*Leprdb*/J , Db/db mice	B6.BKS (D)-*Leprdb*/J , Db/db mice
N=8	**N=8**	**N=8**
Surgically-induced OA Right Knee (MMTL+ MCL Surgery)	**Surgically-induced OA Right Knee** (MMTL+ MCL Surgery)	**Surgically-induced OA Right Knee** (MMTL+ MCL Surgery)
Treatment: -	**Treatment:** DMSO vehicle in PBS	**Treatment:** Rapamycin in DMSO (2mg/kg body weight)

3.2.5 GLUCOSE DETERMINATION

For glucose measurement, mice were fasted for 16 h, and blood was collected from the tail vein. Fasted glucose levels were monitored every two weeks. Blood glucose was measured using a FreeStyle Freedom Lite Glucometer (Abbott).

3.2.6 QUANTIFICATION OF INSULIN

Blood samples were collected from sacrificed mice by cardiac puncture and plasma was separated using BD Microtrainer PST tubes with Lithium Heparin. Insulin levels were determined in plasma samples from all groups at the end of the study, using an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc, USA).

3.2.7 LIVER HISTOLOGY

Liver tissues were fixed in formalin, dehydrated and embedded in paraffin, and 4-µm-thick sections were stained with Hematoxylin-Eosin staining (H&E).

3.2.8 PROTEIN ISOLATION AND WESTERN BLOTTING

Liver from db/db-Vehicle mice and db/db-Rapamycin mice was collected at the time of sacrifice and was immediately frozen in liquid nitrogen and stored at -80ºC until use. Liver was pulverized in a liquid nitrogen-cooled freezer (Microdismembrator 200, Retsch®) at the rate of maximum impact frequency (25Hz, 5 minutes). The proteins were resuspended in lysis buffer (6M urea/2%SDS buffer) and mixed for 1 hour at room temperature in an orbital shaker. The homogenate was centrifuge and the supernatant was recovered. The protein concentrations in the supernatant were determined using a bicinchoninic acid reagent assay (BCA). The concentrated samples were then adjusted to equal volumes before resolution on 4–20% gels (Invitrogen). Protein was

transferred onto nitrocellulose membranes (Invitrogen) and then, blocked with 5% dry milk in Tris buffered saline–Tween (TBST). The membranes were incubated with antibodies for phospho-AMPK (Thr172) (1:1000) and phospho-rpS6 (Ser 235/236) (1:2000) (Cell Signaling Technology, Beverly, MA; 2535, 4858, respectively), GAPDH (1:10000, Invitrogen) and α-tubulin (1:2000, Sigma; T9026). Then, the membranes were incubated with horseradish peroxidase–conjugated anti-mouse IgG (Cell Signaling Technology) for 1 hour. Afterward, the membranes were washed 3 times with TBST and developed using an enhanced chemiluminescent substrate from Pierce. The intensity of the bands was analyzed by using TotalLab (TL120) software.

3.2.9 HISTOLOGICAL ANALYSIS OF MOUSE KNEE JOINTS

Mice knee joints from the three groups were harvested. The joints were fixed in formalin for 24 hours, decalcified solution (Decalc™, HistoLab, 00601) for 6 hours, and then embedded in paraffin. Serial sagittal sections (4µm) were cut, stained with Safranin O-fast green, and examined for histopathological changes using the OARSI semiquantitative scoring system (Glasson et al, 2010). In this system the scores are defined as follows: 0=normal cartilage, 0.5=loss of proteoglycan with an intact surface, 1=superficial fibrillation without loss of cartilage, 2=vertical clefts and loss surface lamina (any % or joint surface area), 3=vertical clefts/ erosion to the calcified layer lesion for 1–25% of the quadrant width, 4=lesion reaches the calcified cartilage for 25–50% of the quadrant width, 5=lesion reaches the calcified cartilage for 50–75% of the quadrant width, 6=lesion reaches the calcified cartilage for >75% of the quadrant width.

3.2.10 HISTOLOGICAL ANALYSIS OF INFLAMMATION

Synovial inflammation was examined using a specific grading system (Krenn et al, 2002). Three parameters, hyperplasia/enlargement of synovial lining cell layer, activation of resident cells/synovial stroma and inflammatory cell infiltration were graded as absent (grade 0), slight (grade 1), moderate (grade 2) or severe (grade 3).

3.2.11 IMMUNOHISTOCHEMISTRY

Sections from paraffin-embedded joints were first deparaffinized in the xylene and rehydrated in graded ethanol and water. For antigen unmasking, sections in 10 mM sodium citrate buffer (pH 6.0) were heated in a microwave oven and kept at 80–85°C for 1.5 minutes. Slides were cooled for 20 minutes at room temperature after antigen unmasking. After washing with phosphate buffered saline (PBS), sections were blocked with 5% serum for 30 minutes at room temperature. Then, sections were incubated with antibodies for MMP-13 (1:50) (Abcam, Cambridge, MA, ab84594)

and LC3 (1:3200), (Cell Signaling Technology, Beverly, MA; 3868) overnight at 4°C. After washing with PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody for 30 minutes at room temperature, and then incubated using Vectastain ABC-AP alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 30 minutes. Slides were washed, and sections were incubated with 3,3´-diaminobenzidine (DAB) substrate for 3-10 minutes.

3.2.12 Quantification of MMP-13-positive cells

In synovium from Lean, db/db-Vehicle and db/db-Rapamycin, the total number of MMP-13-positive cells was counted in two independent areas of each section.

3.2.13 Mouse Cytokine Magnetic 10-Plex Panel

Blood samples were collected from killed mice by cardiac puncture and plasma was separated using BD Microtrainer PST tubes with Lithium Heparin. Cytokines levels were determined in serum samples using a Mouse Cytokine Magnetic 10-Plex Panel for simultaneous quantitative determination of GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70 and TNF-α (Invitrogen; LMC0001M).

3.2.14 Cartilage cellularity

Knee joint sections were stained with Hematoxylin and Eosin (H&E). In cartilage from knees with experimental OA, three pictures were taken under 10x magnification, representing the centre of the femoral condyle that is not covered by the menisci as well as the medial and lateral femoral condyles. The total number of cells in each section was counted (Taniguchi et al, 2009).

3.2.15 Statistical analysis

To test for normal distribution of the data, we used the Kolmogorov-Smirnov test. In general, the data sets follow a normal distribution. In the cases where the number of samples was too small to be tested accurately, we assumed the data follow a normal distribution. Statistically significant differences between 2 groups were determined by Student's t-test. Statistically significant differences between multiple groups were determined analyzing variance (ANOVA) in conjunction with multiple comparison test. Data are presented as the mean (lower level, upper level) (ll, ul) 95% confidence interval (CI). The data analysis and statistical inference was performed by using Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

3.3 RESULTS

3.3.1 AUTOPHAGY ACTIVATION BY RAPAMYCIN IMPROVES METABOLIC PARAMETERS IN DB/DB MICE

Diabetic (db/db) mice are obese, hyperglycemic and hyperinsulinemic with markedly insulin resistant. At the end of our study, body weight of db/db mice was 32% higher than Lean mice (47,5±1,254 g vs 32,25±0,70 g, $p<0.0001$). Importantly, Rapamycin treatment significantly reduced body weight in db/db mice (Figure 3.3A). Furthermore, a marked increase in fasted blood glucose levels and plasma insulin levels were observed in db/db mice compared to Lean group. However, db/db mice subjected to experimental OA and treated with Rapamycin exhibited a significant reduction in fasted blood glucose levels and plasma insulin levels, indicating that Rapamycin treatment improves metabolic parameters in db/db mice (Figure 3.3B). In addition, a dramatic reduction in lipid accumulation is observed in liver tissue of Rapamycin treated animals (Figure 3.3C). To demonstrate target engagement of Rapamycin treatment, phosphorylation of AMP activated protein Kinase (p-AMPK) and phosphorylation of ribosomal protein S6 (p-prS6), a direct target of mTOR, were determined. Indeed, Rapamycin significantly increased p-AMPK expression and significantly reduced p-prS6 expression in liver tissue (Figure 3.3D).

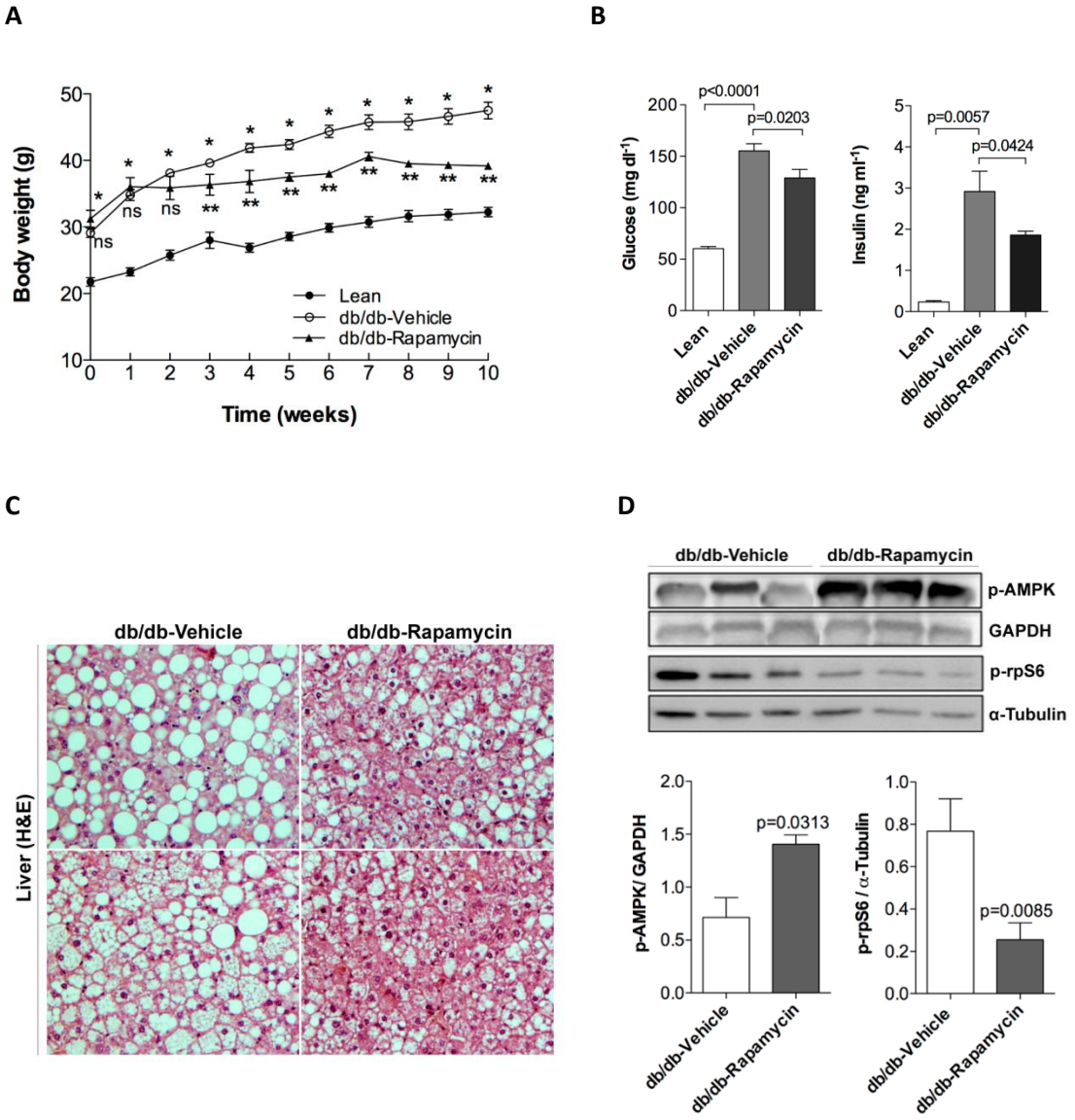

Figure 3. 3 **- Autophagy activation by Rapamycin improves metabolic parameters in db/db mice. A,** Body weight of Lean, db/db-Vehicle and db/db-Rapamycin mice was evaluated every week during 10 weeks. Values are the means (ll, ul) 95% CI of eight mice in each experimental group. *p<0.0001 vs. Lean mice and **p (t_0:p=0.4482, t_1:p=0.2376, t_2=0.0673, t_3:p=0.0382, t_4:p=0.0117, t_5:p=0.0004, t_6:p<0.0001, t_7:p=0.0030, t_8:p=0.0009, t_9:p=0.0003, t_{10}:p=0.0002) vs. db/db-Vehicle mice. **B,** Fasted blood glucose levels were determined after three measurements during the study. Values are the means (ll, ul) 95% CI of eight mice in each experimental group. Fasted plasma insulin levels were determined at the end of the study. Values are the means (ll, ul) 95% CI of eight mice in each experimental group. **C,** Liver sections from db/db-Vehicle and db/db-Rapamycin mice (N=8 each/group) were analyzed by staining with H&E. Representative images of liver sections from two mice per group. **D,** Liver protein extracts from db/db-Vehicle mice and db/db-Rapamycin mice. Phosphorylation of AMP activated protein kinase (AMPK) and ribosomal protein S6 (rpS6) was determined by Western blotting. Densitometric analysis of p-AMPK and p-rpS6. GAPDH and α-Tubulin were employed as a loading control. Values are the means (ll, ul) 95% CI of 5 mice in each experimental group.

3.3.2 DIABETES ACCELERATES EXPERIMENTAL OA IN DB/DB MICE

To investigate the influence of T2D in OA progression, Lean mice and db/db mice were subjected to experimental OA by transection of the medial meniscotibial ligament and the medial collateral ligament in the right knee. Left knee joints were used as an internal control. 10 weeks after surgery, right and left knee joints were evaluated by Safranin O staining. The results showed OA-like changes in the right knee in both Lean and db/db mice (Figure 3.4 A,B). A significant increase in cartilage damage was found in OA-db/db mice compared to OA-Lean mice (Figure 3.4C), while control knee joints did not show histopathological cartilage changes. These results suggest first, that diabetes accelerates cartilage damage after experimental OA and second, that this effect is associated to metabolic disturbances of diabetic mice.

3.3.3 mTOR INHIBITION PREVENTS DIABETES-ACCELERATED EXPERIMENTAL OA

To evaluate if pharmacological mTOR inhibition has beneficial effects on diabetes-accelerated OA, db/db mice were subjected to experimental OA (N=16). Db/db-Vehicle mice exhibited OA-like changes with loss of proteoglycans and structural changes in the surface lamina. On the other hand, db/db-Rapamycin mice only showed a focal loss of proteoglycans and small structural changes in the articular cartilage (Figure 3.4A,B), associated to a reduced histological OA score (Figure 3.4C). These results demonstrate that mTOR inhibition prevents diabetes-accelerated experimental OA in mice.

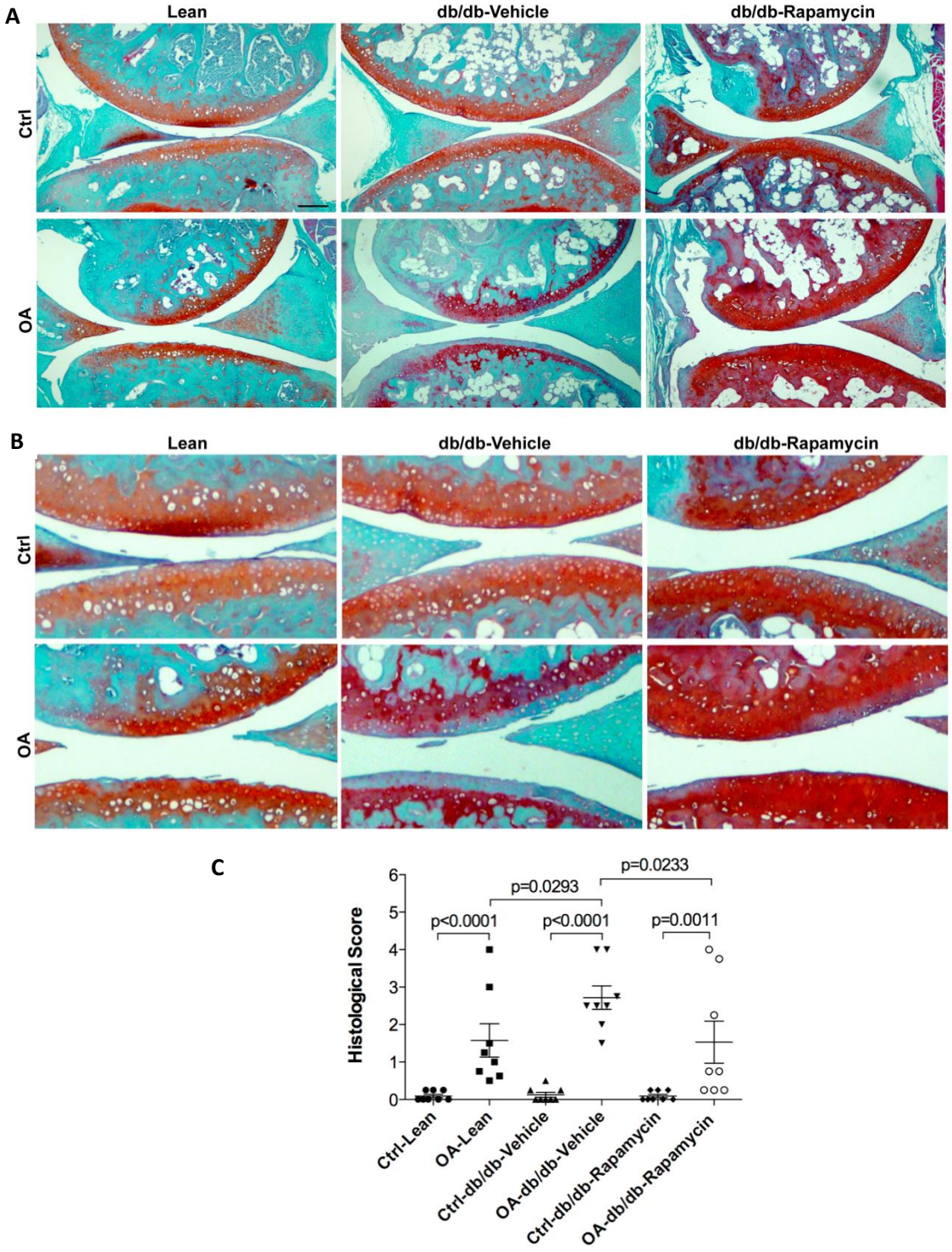

Figure 3. 4 **- Diabetes-accelerated experimental osteoarthritis is prevented by mTOR inhibition.** Nine-week-old male db/db mice (B6.BKS (D)-*Leprdb*/J, N=16) and the respective background strain mice (C57BL/6J-non-diabetic lean mice, N=8) were subjected to experimental osteoarthritis (OA) in the right knee (OA). The left knee was not subjected to surgery and was used as a control (Ctrl). Three experimental mice groups were performed; Lean mice, db/db-Vehicle mice and db/db-Rapamycin mice. Each experimental group included eight mice. **A-B,** Knee joints were analyzed by staining with Safranin O. Scale bar 100µm. Magnification: 4x and 10x. **C,** Cartilage histological scores of Lean, db/db-Vehicle and db/db-Rapamycin mice. Values are the means (ll, ul) 95% CI of eight mice in each experimental group (each point represents an individual mice).

3.3.4 RAPAMYCIN REDUCES SYNOVIUM INFLAMMATION IN DIABETES-ACCELERATED EXPERIMENTAL OA

Inflammation is important in OA and T2D pathophysiology (Donath & Shoelson, 2011), although how bioenergy sensors link metabolism with inflammatory processes to regulate joint integrity is unknown. We investigated how diabetes affects the synovium of db/db mice. In this sense, a significant increase of synovial inflammation is observed after surgery in both Lean and db/db mice, although a greater inflammation score is found in db/db mice. Interestingly, Rapamycin treatment reduced synovial inflammation in db/db while no signatures of histopathological changes were observed in the Control knee joints (Figure 3.5A,B). These results suggest that the beneficial effect of mTOR inhibition in joints might be mediated by a reduction in synovium inflammation.

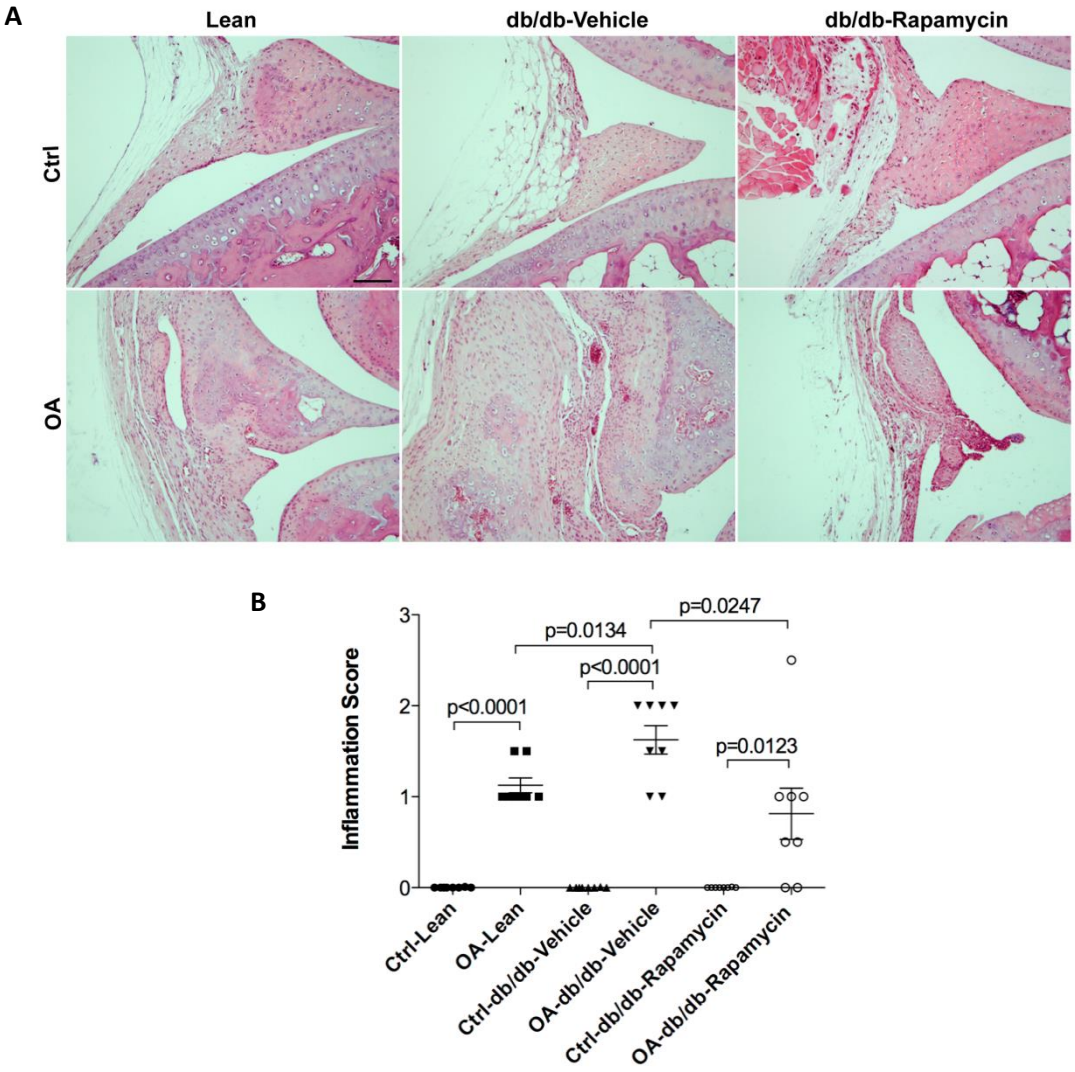

Figure 3. 5 **- Effect of Rapamycin on synovial inflammation in experimental OA. A,** Synovium from Lean, db/db-Vehicle and db/db-Rapamycin mice (Ctrl and OA, N=8 each group) was analysed by staining with Hematoxylin Eosin (H&E). Scale bar 100µm. Magnification: 10x. **B,** The histological score for inflammation was significantly increased in OA-db/db-Vehicle mice compared to OA-Lean mice and significantly decreased after Rapamycin treatment. Values are the means (ll, ul) 95% CI of eight mice in each experimental group (each point represents an individual mice).

3.3.5 RAPAMYCIN REDUCES THE EXPRESSION OF CATABOLIC FACTORS INVOLVED IN CARTILAGE LOSS AND INFLAMMATION IN DB/DB MICE

To evaluate the mechanism of action of diabetes-accelerated cartilage degradation in db/db mice, immunohistochemistry analysis was performed for MMP-13, an important OA biomarker involved in cartilage degradation (Takaishi et al, 2008). The results showed a significant increase in the expression of MMP-13 in OA db/db-Vehicle mice, predominantly in the synovium area. Importantly, Rapamycin treated OA db/db mice showed a significant reduction in the expression of MMP-13, indicating a potent anti-catabolic effect of mTOR inhibition in db/db mice (Figure 3.6A,B).

To go further in the explanation of the protective effects of Rapamycin, proinflammatory cytokine levels were evaluated in plasma from db/db-Vehicle mice and db/db-Rapamycin mice. The results showed a significant reduction in serum levels of active interleukin 12 (IL-12p70), a proinflammatory cytokine (Radstake et al, 2004), in db/db-Rapamycin mice compared to db/db-Vehicle mice ($p<0.01$) (Figure3.6C), suggesting a potential common link between T2D and OA inflammatory mechanisms.

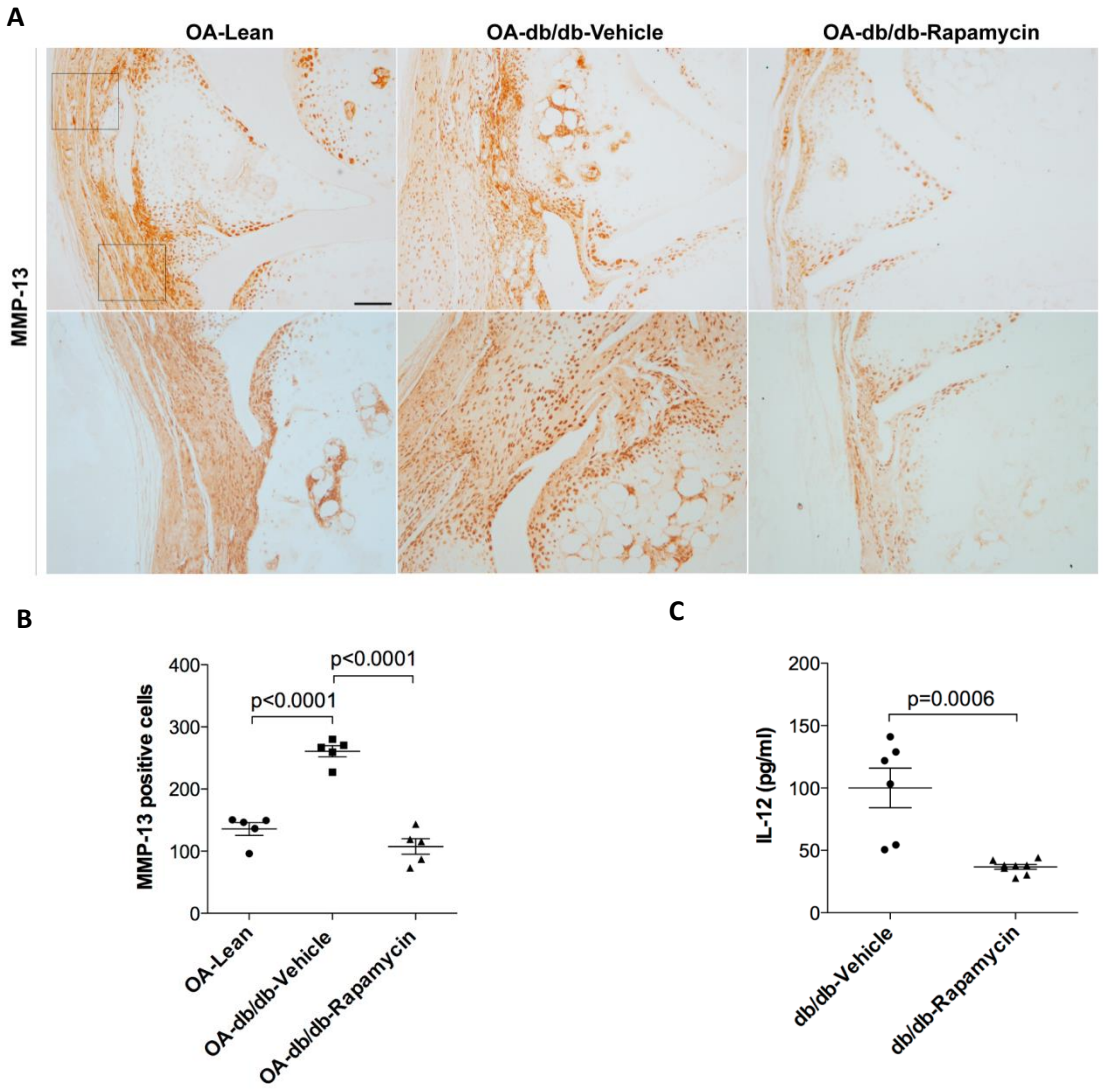

Figure 3. 6 - **Rapamycin reduces the expression of catabolic factors implicated in the joint damage in db/db mice. A,** Knee joints from Lean, db/db-Vehicle and db/db-Rapamycin mice were collected 10 weeks after surgery (N=5 each/group). Sections were analysed by immunohistochemistry for MMP-13. Two sections from one animal are presented. Scale bar 100µm. Magnification: 10x. **B,** Quantitative analysis of MMP-13 positive cells. Values are the means (ll, ul) 95% CI of five mice in each experimental group (each point represents the mean of two independent areas from each mice). **C,** Quantitative analysis of Interleukin-12 (IL12p70) in serum from db/db-Vehicle and db/db-Rapamycin mice. Values are the means (ll, ul) 95% CI of six mice in db/db-Vehicle group and eight mice in db/db-Rapamycin group (each point represents individual mice).

3.3.6 RAPAMYCIN MAINTAINS CHONDROCYTE HOMEOSTASIS IN DB/DB MICE

Autophagy, a key homeostatic mechanism in articular cartilage, is reduced in chondrocytes from OA diabetic patients (Ribeiro et al, 2016). To confirm this evidence, LC3, a main marker for autophagosome formation (Mizushima et al, 2010), was evaluated. The results showed that LC3 staining was reduced after experimental OA in db/db-Vehicle mice. Remarkably, mTOR inhibition by Rapamycin maintains LC3 expression in db/db mice knee joints subjected to experimental OA (Figure 3.7A).

Chondrocyte cellularity in mouse knee joints was significantly reduced in chondrocytes in OA-db/db-Vehicle mice compared to OA-Lean mice. Preservation of cellularity is observed in Rapamycin treated mice compared to OA db/db-Vehicle mice (Figure 3.7B).

Together, these data suggest that cartilage homeostasis is compromised in db/db mice subjected to experimental OA, and that autophagy activation protects against chondrocyte loss.

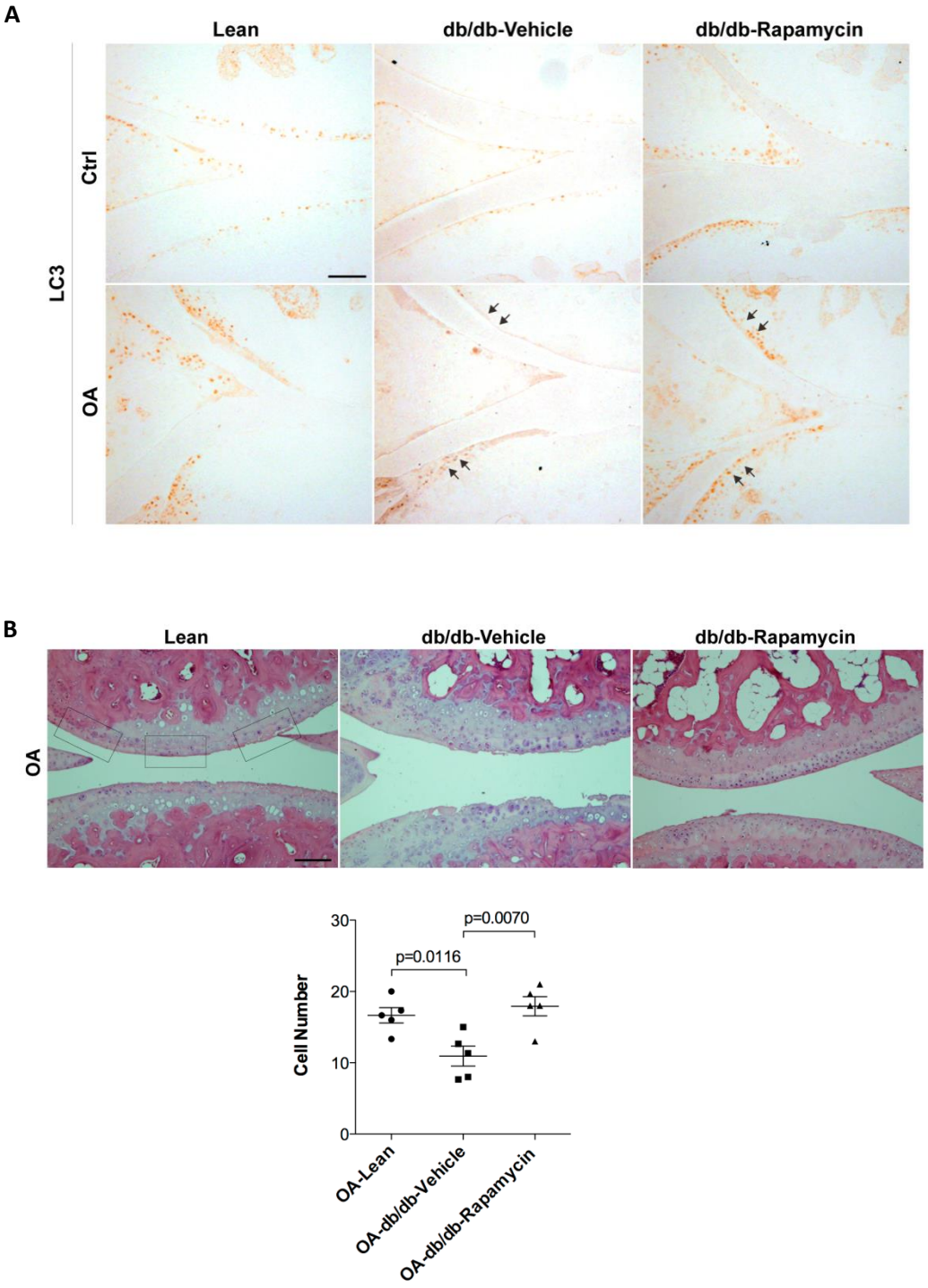

Figure 3. 7 - **Rapamycin maintains chondrocyte homeostasis in db/db mice. A,** Knee joint from Lean, db/db-Vehicle and db/db-Rapamycin mice with experimental OA (N=3 each/group) were analyzed by immunohistochemistry for LC3, a marker for autophagosome formation and autophagy activation. Scale bar 100µm. Magnification: 10x. **B,** Quantitative analysis of cell number in OA-Lean, OA-db/db-Vehicle and OA-db/db-Rapamycin mice (N=5 each/group). Values are the means (ll, ul) 95% CI of five mice in each experimental group (each point represents the mean of three independent areas for each mice).

3.4 DISCUSSION

Metabolism influences dramatically health outcomes, including articular cartilage and synovium function. However, the consequences of metabolic complications of diabetes in OA are not well understood. There are evidences indicating that failure of homeostasis mechanisms such as autophagy, leads to alterations in articular cartilage that ultimately cause joint damage and OA (Carames et al, 2010). Based on these concepts, we previously demonstrated that high glucose and high insulin reduced autophagy in human chondrocytes and accelerated cartilage degradation through regulation of Akt/mTOR signaling pathway. We found that pharmacological activation of autophagy attenuates the catabolic effects of high glucose and high insulin in human chondrocytes. Our findings suggest that reduced autophagy might be one of the mechanisms responsible for the cartilage degradation observed in T2D patients (Ribeiro et al, 2016).

In the present study, we investigate the implication of T2D in OA development by using a genetic diabetes mice model. These mice have a mutation on the leptin receptor and lack leptin signaling, which leads to hyperphagia, persistent hyperinsulinemia, hyperglycemia and obesity (Srinivasan & Ramarao, 2007). Db/db mice exhibit metabolic disturbances similar to those observed in humans with T2D, being a representative model to study diabetes and the associated metabolic complications (Lin & Sun, 2010). Human metabolic diseases have been linked to mTOR dysregulation (Tsang et al, 2007). We evaluated how systemic Rapamycin treatment, an autophagy inducer by mTORC1 inhibition, influences metabolic phenotype of db/db mice. Rapamycin significantly improved body weight gain, fasted blood glucose levels and serum insulin levels in db/db mice. To investigate the link with the targets potentially responsible for these beneficial effects, we evaluated the activity of AMPK, a key energy sensor that regulates cellular metabolism to maintain energy homeostasis and p-rpS6, a direct target of mTOR (Kim et al, 2011). Consistent with established downstream activity, Rapamycin reduced p-rpS6 while increased the phosphorylation of AMPK. The data is a confirmation of the pharmacological activity of Rapamycin in our model, related to AMPK response to energy stress probably through inhibition of the mTORC1 pathway and autophagy activation (Habib, 2011).

To study the connection between T2D and OA progression, we performed experimental OA in Lean and db/db mice. Our results showed that articular cartilage of OA-db/db mice developed more severe OA-like changes in articular cartilage compared to OA-Lean mice. Notably, synovium inflammation and thickening was found in the joints of OA-db/db mice. Joint inflammation was accompanied with an increase in MMP-13, predominantly in the synovial membrane. Together, these

results support the concept that abnormal glucose metabolism, primarily associated with high levels of glucose and insulin accelerates experimental OA in db/db mice.

The maintenance of autophagy is essential to preserve cartilage integrity. Decreased autophagy and increased chondrocyte death now established features of OA (Hwang & Kim, 2015; Musumeci et al, 2015b). In this sense, articular cartilage from diabetic mice displayed a reduction of LC3, and a decrease in cartilage cellularity, which suggest that joint damage observed in db/db mice might be due to defective autophagy which leads to an imbalance between the catabolic and anabolic pathways.

Since decreased in autophagy could be one of the mechanisms responsible for the faster cartilage damage observed in diabetes, we evaluated whether pharmacological autophagy activation by Rapamycin could confer protection to joints. Indeed, Rapamycin treatment reduces the severity of experimental OA in C57BL/6J mice and prevents OA progression, consistent with previous observations (Carames et al, 2012a; Matsuzaki et al, 2014; Takayama et al, 2014; Zhang et al, 2015). Our results show that Rapamycin attenuates the severity of experimental OA in db/db mice, demonstrated by the diminished OA cartilage score in the treated mice. Moreover, Rapamycin treatment dramatically reduces synovial inflammation, the expression of MMP-13 and circulating IL-12 levels were significantly reduced in response to Rapamycin. Previous studies demonstrated an increase in IL-12 levels in serum from patients with T2D (Wegner et al, 2008) and in mice subjected to high fat diet and posttraumatic arthritis (Louer et al, 2012), suggesting a potential common inflammatory pathway for diabetes and OA (Aiello et al, 2015). In fact, OA cartilage from diabetic patients is more reactive to pro-inflammatory stress than OA cartilage from non-diabetic patients, suggesting that diabetes increases inflammation in OA (Laiguillon et al, 2015). Our results are consistent with previous findings where autophagy activation can prevent inflammatory responses (Mobasheri et al, 2015; Salminen et al, 2012).

Furthermore, Rapamycin increases LC3 expression and maintains the chondrocyte number in db/db mice**.** These findings together with our previous observations suggest that Rapamycin treatment reduces joint damage in diabetic mice with experimental OA by both normalizing altered metabolic parameters of T2D and by inducing autophagy.

It is important to note that db/db mice are not only diabetic, but they have increased body weight and obesity, which is also an important risk factor for OA (Louer et al, 2012; Srinivasan & Ramarao, 2007). Since we performed experimental OA only in right knee, left knee was employed as a control to evaluate the direct effect of obesity in the joint in this time frame. We observed

increased cartilage damage and synovial inflammation in the OA knee, while control knee did not show any pathological changes. These results suggest that body weight is not sufficient to accelerate experimental OA in db/db mice. One of the limitations of this study is that contralateral limbs of experimental OA were used as control joints as opposed to sham surgery joints. It is possible that an alteration of joint load can be developed as a result of experimental OA in the joint.

To conclude, our study identifies a promising therapeutic approach to prevent cartilage degradation observed in T2D patients. However, Rapamycin, has some effects, which limits its use for preventive strategies in humans (Lamming et al, 2013). Further studies are needed to identify new drugs that can activates autophagy and modify the OA progression in diabetic patients. This knowledge could leads to an improvement of clinical care in OA patients with metabolic diseases.

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CHAPTER 4

DISCUSSION AND CONCLUSION

Considering the current increase in human longevity, a concurrent increase in age-related diseases such as OA is very likely to occur. The burden of OA includes pain, functional limitation in daily activities, and locomotor impairment, resulting in a huge impact on quality of life as well as on life expectancy (Hunter et al, 2014). Because efficient therapies for OA are not currently available, identifying individuals with a high risk to develop OA is crucial in the attempt to prevent OA development and progression. Nowadays, OA is considered a multifactorial disease with many risk factors associated. One of them is diabetes, which has recently emerged as one of the possible diseases linked to an earlier OA onset and exacerbation of OA development. Despite the growing data supporting this association, the impact of diabetes on connective tissue physiology is likely underestimated due to the high prevalence of undiagnosed diabetic patients, contributing to its under-appreciation when compared to other factors like mechanical stress.

As described in Chapter 1, some mechanisms have been proposed as possible links between these two diseases. However, the mechanisms identified as relevant are still few and its pathophysiology still not fully understood. It is recognized that diabetes induces catabolic and inflammatory processes, however, the exact mechanisms involved are still largely unknown. The identification and better understanding of this is crucial to identify possible therapeutic targets. Furthermore, possible modulation of these targets can result in new effective therapeutic strategies that can prevent or halt diabetes-associated OA development and progression.

The general aim of this work is to identify pathological mechanisms involved in the association between both diseases. The poor regenerative capacity of articular cartilage makes homeostatic mechanisms, such as autophagy, key tools in maintaining normal tissue function. In this context, and considering that autophagy is dysregulated both in OA and in diabetes, one goal of this work is to evaluate the role of diabetic conditions in modulating autophagy in chondrocytes and joint tissues, and its relation with articular cartilage and joint damage. Understanding whether pharmacological autophagy activation can counteract the deleterious effects of diabetes in chondrocytes, articular cartilage and the joint, and thus, whether autophagy modulation can constitute a therapeutic target to prevent diabetes-associated OA development and progression, is a logical consequence of those aims and an important secondary goal of this study. Using both *in vitro* and *in vivo* models, we were able to positively address these aims.

Specifically and in brief, the results presented in chapter 2 show that:

- In the presence of high glucose and increasing concentrations of insulin, the human chondrocytic cell line, TC28a2, primary human chondrocytes and human cartilage explants exhibit a decreased expression of LC3, while downstream targets of mTORC1, namely the

activity of the rpS6 kinase, assessed as the phosphorylated levels of its target protein rpS6, were increased, together indicating a reduction in autophagic activity. This decreased autophagy seems to be mediated by increased activity of the Akt/mTOR signaling pathway.

- High insulin concentrations are sufficient to decrease autophagy in human chondrocytes.
- The same diabetic conditions (high glucose and high insulin concentrations) also lead to loss of proteoglycans and a significant increase in MMP-13 and IL-1β expression, suggesting a catabolic effect in chondrocytes and articular cartilage.
- Pharmacological activation of autophagy, by mTOR inhibition with Rapamycin, prevents the decrease in autophagy and the increase in inflammation, observed when chondrocytes were exposed to diabetic conditions.
- Chondrocytes from diabetic OA patients exhibit a decreased expression of LC3-II and an increase in rpS6 phosphorylation, compared to chondrocytes from non-diabetic OA patients. These results suggest that chondrocytes from diabetic OA patients display decreased basal levels of autophagy accompanied by increased activity of the mTORC1 pathway, compared to non-diabetic OA chondrocytes.

These *in vitro* results encouraged us to further pursue our research goals, this time using an *in vivo* model. The results of the *in vivo* study are detailed in chapter 3 and show that:

- Vehicle-treated db/db mice exhibit an increase in cartilage damage and in synovial inflammation, an up-regulation of OA biomarkers, such as MMP-13, and a decrease in cell number. Furthermore, a decrease in LC3 staining was also detected in vehicle-treated db/db mice compared to lean mice, both subjected to OA induction. These findings show that, compared to lean mice, db/db mice develop an accelerated form of surgically-induced OA that is accompanied by impairment in autophagy, suggesting that T2D compromises autophagy and accelerates experimental OA.
- Rapamycin, a well-recognized pharmacological autophagy activator that acts through mTOR inhibition, improves metabolic parameters such as body weight, fasting blood glucose, and insulin levels in OA-db/db mice. In addition, Rapamycin reduces the histological OA score, synovial inflammation, the expression of catabolic factors involved in cartilage degradation (MMP-13) and in inflammation (IL-12), preserves chondrocyte viability and increases LC3 staining in joint tissues, in OA-db/db mice. Together, these results suggest that pharmacologic mTOR inhibition, and the consequent autophagy activation, reduces the severity of experimental OA in db/db mice.

Following the proposed research goals, this work supports the existence of an association between diabetes and OA, observable in both *in vitro* and *in vivo* models.

Highlighting the importance of translating *in vitro* hypothesis into *in vivo* models, other groups have also been using T2D mice to confirm the link between T2D and OA, achieving similar results that strengthen our findings (Onur et al, 2014).

Diabetic conditions damage several tissues through the body, including the musculoskeletal system, namely bones, tendons and ligaments, synovium, and articular cartilage, all involved in OA. The effects of hyperglycemia in articular cartilage were described in the introduction, but effects in other joint tissues are less studied. For instance, in tendons, it seems that hyperglycemia reduces PGs levels contributing to increased stiffness and leading to tendon pathology observed in diabetes (Burner et al, 2012). In bones, hyperglycemia can disrupt the skeletal homeostasis by interfering in the equilibrium between osteoblast (bone forming cells) and osteoclast (bone resorbing cells) activity, altering bone strength and consequently impairing bone health (Garcia-Hernandez et al, 2012; Kang et al, 2015a; Sanguineti et al, 2008). Thus, further studies evaluating the effects of hyperglycemia in different joint tissues should also be an important issue to consider in order to understand the mechanism underlying this association.

So far, diabetic complications seem to be mainly due to hyperglycemia. So, current therapies that mainly focus on achieving glucose homeostasis should decrease or prevent those complications. However, this does not always occur. A possible explanation can be the inadequate control of glucose levels, leading to persistent hyperglycemia that causes or exacerbates diabetic complications. However, in cases of good control of glucose levels, the involvement of other factors, beyond hyperglycemia, can be another explanation. Another important hallmark of T2D is hyperinsulinemia that occurs in both early and late stages of the disease. However, only few studies have addressed the effects of hyperinsulinemia in joint tissues. Regarding the synovium, a potential protective and anti-inflammatory role of high insulin concentration was proposed. In human fibroblast-like synoviocytes (FLS), insulin decreased the expression of cartilage degradative enzymes (MMP1,13 and ADAMTS4) induced by TNF-α (Hamada et al, 2016). In contrast, in distinct cells, negative effects of hyperinsulinemia have been reported. Indeed, high glucose and high insulin environment suppressed osteoclastogenesis, impaired osteoclast function, and caused functional loss of osteoblasts, suggesting that a disruption of the bone metabolism can be mediated by both factors (Freude et al, 2012; Xu et al, 2013). Although the effects of hyperinsulinemia remain poorly understood, it seems that both hyperglycemia and hyperinsulinemia, separately or in conjunction, can be important factors when evaluating diabetic complications. For instance, the negative effects of

hyperinsulinemia were also observed in other tissues. It increases progression of retinopathy, compromises respiratory health and promotes cardiomyopathy (Madonna et al, 2014; Roysarkar et al, 1993; Singh et al, 2016). In addition, a recent work from our group (unpublished results) and the results presented in this thesis also show the negative effects of hyperinsulinemia in articular chondrocytes: it can induce inflammatory and catabolic responses and decrease autophagy. Together these results shed light on a possible factor contributing to the prejudicial effects of diabetes in several tissues, including articular cartilage. Therefore, diabetic complications, including OA, can be developed not exclusively due to hyperglycemia, but factors such hyperinsulinemia can also play an important and harmful role. Further studies are needed to prove and clarify this emerging notion. However, if this proves to be correct, treatments to achieve normal levels of both glucose and insulin would be preferred as they would allow a reduction in diabetic complications. Nevertheless, if this new concept is correct, another issue arises: if hyperinsulinemia is a harmful factor, Type 1 Diabetic patients and T2D patients at late stages that require insulin therapy, may have increased diabetic complications. This is also an important issue that should be explored and interpreted with caution.

Inflammation is another important issue to consider when evaluating the interaction between both diseases. In fact, a possible association between diabetes and inflammation in chondrocytes has been suggested (Laiguillon et al, 2015); Rufino et al, 2014). Moreover, recent evidence shows that AGEs induce inflammatory responses in synovial cells (Chen et al, 2016; Lin et al, 2016). Since AGE accumulation occurs in diabetes, this can suggest that diabetes may have an impact on OA through induction of AGE-mediated synovial inflammation. However, further studies directly assessing the effects of hyperinsulinemia and hyperglycemia in synovial membrane are essential to clarify the validity of this hypothesis. In fact, the results obtained from the *in vivo* study presented in this thesis also highlight the importance of inflammation in the association between both diseases. After surgical OA induction, db/db mice exhibited a higher increase in synovial inflammation and in cartilage degeneration, compared to lean mice. Moreover, after Rapamycin treatment, an improvement in metabolic parameters and a reduction in synovial inflammation and in cartilage degradation were observed. Together, these observations reinforce the notion that T2D induces a chronic inflammatory state, intensifying and sustaining the increased levels of inflammation that can affect cartilage integrity.

Considering the goals of this thesis, autophagy is proposed as a possible mechanism involved in diabetes-associated OA and its modulation as a way to prevent the rapid cartilage degradation observed in those patients. In fact, it was suggested that autophagy may play an important role in the pathophysiology of other diabetic complications such as nephropathy, neuropathy, retinopathy, and cardiomyopathy (Kobayashi & Liang, 2015; Li et al, 2015; Tagawa et al, 2016). Consequently, the

modulation of autophagy to prevent these complications has also been suggested. For instance, autophagy activation can prevent diabetic nephropathy (Xu et al, 2015), and diabetic neuropathy (Qu et al, 2016; Sekiguchi et al, 2012). This activation also protects against cardiac apoptosis and improves cardiac function in diabetic mice, contributing to diabetic cardiomyopathy prevention (He et al, 2013). Moreover, the inhibition of autophagy worsens the impaired osteoblastic cell survival mediated by high glucose, suggesting that maintaining osteoblast autophagy may be a strategy to prevent diabetes-related osteopenia (Bartolome et al, 2013). On the other hand, it seems that autophagy contributes to increase brain injury in diabetic conditions so, in this case, autophagy inhibition would have protective effects against brain injury (Wei et al, 2013).

Additionally, the results presented in this thesis also suggest that the mTOR/Akt signaling pathway plays a role in autophagy impairment. However, many other mechanisms may also be involved. In this sense, further studies are needed to identify such mechanisms and to explore their potential as therapeutic targets to treat and prevent diabetes-associated OA. Among others, NF-κB, AMPK and SIRT1 may also be considered as they are able to sense diabetic conditions and can also modulate autophagy.

As previously mentioned, it seems that both hyperglycemia and hyperinsulinemia, separately or in conjugation, activate NF-κB in chondrocytes. NF-κB, a family of transcription factors with a critical role in several processes, is considered a master regulator of inflammatory responses, having an important role in OA. In articular cartilage, NF-κB regulates the expression of different matrix degrading enzymes, cytokines and inflammatory mediators, influencing the remodeling of ECM proteins, contributing to cartilage degradation and joint inflammation (Marcu et al, 2010). There is some evidence suggesting a crosstalk between NF-κB and autophagy. However, the exact role of NF-kB signaling in autophagy in chondrocytes remains unknown. In fact, in cancer cells, NF-κB activation inhibits the induction of autophagy promoted by TNF-α (Djavaheri-Mergny et al, 2006). Moreover, both *in vivo* and *in vitro* studies demonstrated that NF-κB inhibition increases levels of autophagy in the hippocampus of rats and in porcine granulosa cells (Gao et al, 2016; Su et al, 2015). So, it is possible that the increased activity of NF-κB mediated by diabetic conditions in chondrocytes can imply a decrease in autophagy. Further studies exploring the potential role of NF-κB in chondrocyte autophagy and how activation of one pathway leads to inhibition of the other are needed.

AMPK is a serine/threonine protein kinase that integrates nutritional and hormonal signals with an important role in cellular energy homeostasis and metabolism. In fact, reduction in AMPK activity with acute hyperglycemia was observed in liver, muscle and kidney (Kraegen et al, 2006; Lee et al, 2007), and with increased insulin concentration in isolated rat hearts (Gamble & Lopaschuk,

1997). Therefore, dysregulation of AMPK activity has been associated with many diseases like diabetes and, more recently, with OA. Normal articular chondrocytes express AMPK and it is constitutively phosphorylated. However, human knee OA chondrocytes and human knee OA cartilage show a decrease in AMPK expression and phosphorylation. In chondrocytes, the decreased phosphorylation of AMPK increases apoptosis and the catabolic responses induced by inflammatory cytokines. Moreover, pharmacological AMPK activators attenuate catabolic responses induced by inflammatory cytokines and biomechanical compression injury (Petursson et al, 2013; Terkeltaub et al, 2011). Beyond this anti-inflammatory and anti-catabolic effect, AMPK also regulates anti-oxidative stress capacity in chondrocytes, through its downstream targets, transcriptional coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), and FOXO3a (Zhao et al, 2014). Additionally, when energy is insufficient, AMPK can also induce autophagy direct or indirectly. Directly by ULK complex activation and indirectly through phosphorylation of TSC2 and consequently mTORC1 inhibition, through modulation of the expression of FOXO transcription factors which in turn regulates the Atgs expression, or even through the stimulation of JNK that leads to dissociation of Beclin from Bcl-2, that consequently binds to hVPS34 and activates autophagy (Yao et al, 2016). Indeed, this activation of autophagy through AMPK was demonstrated in chondrocytes (Bohensky et al, 2010). Therefore, as an upstream energy sensor and an autophagy activator, AMPK may be also involved in the decrease in autophagy mediated by hyperglycemia and hyperinsulinemia. Accordingly, in the *in vivo* study presented here, Rapamycin improved metabolic parameters including glucose and insulin levels that were accompanied by an increase in AMPK phosphorylation. Moreover, an activation of autophagy and an attenuation of diabetes-induced joint destruction were also observed, suggesting that AMPK can also be involved in diabetes-induced OA.

Another downstream target of AMPK is SIRT1. Sirtuins are a family of NAD-dependent deacetylases comprising seven members, SIRT1 to SIRT7. Among them, SIRT1 is the most studied one, which plays important roles in several biological functions. Through the deacetylation of substrates including FOXO family members, the tumor suppressor, p53, and NF-κB, it regulates cellular stress response and survival. SIRT1 also acts on PPARγ and PGC-1α and regulates metabolic activities. Through the increase in activity of liver protein kinase B1 (LKB1), an upstream kinase that promotes AMPK activity, it also regulates cellular metabolism (Ng & Tang, 2013). Dysregulation of SIRT1 has been associated not only with OA (Dvir-Ginzberg & Steinmeyer, 2013) but also with other diseases, including T2D (Kitada & Koya, 2013). More specifically, in chondrocytes, SIRT1 promotes cartilage specific gene expression (Dvir-Ginzberg et al, 2008); reduces ionizing radiation-induced senescence phenotype (Hong et al, 2010), produces anti-inflammatory effects by deacetylation and inactivation of NF-κB (Moon et al, 2013), and mediates their survival (Takayama et al, 2009). In fact,

in OA, SIRT1 expression is decreased and this reduction is associated with chondrocyte hypertrophy and cartilage matrix loss – down-regulation of aggrecan and type II collagen, and up-regulation of collagen-X, and ADAMTS5 (Dvir-Ginzberg & Steinmeyer, 2013). In insulin resistant and glucose intolerant states, downregulation of SIRT1 is also observed (Ceolotto et al, 2014; de Kreutzenberg et al, 2010; Frojdo et al, 2011). In addition, SIRT-1 has also been proposed to regulate autophagy. In fact, SIRT-1 can regulate autophagy directly, through deacetylation of Atg proteins such as Atg5, 7 and 8, and indirectly, through deacetylation of FOXO3a and p53 (Ng & Tang, 2013). Thus, SIRT1 activators leading to autophagy activation may also be promising drug candidates to prevent diabetes-associated OA. Further studies will be required to understand if these mechanisms are involved in the impaired autophagy observed in chondrocytes and articular cartilage in diabetic conditions. The detailed identification of these mechanisms seems essential in order to explore the possibility of using them as future new therapeutic targets for this subset of patients.

Additionally, and considering autophagy activation as an option to treat and/or prevent diabetes-associated OA, several autophagy inducers can be tested. Autophagy can be modulated by mTOR-dependent and independent signaling pathways, as described in the Introduction. In this work, because a decrease in autophagy and an increase in mTOR pathway under diabetic conditions were observed in the *in vitro* model, autophagy was induced through mTOR inhibition using Rapamycin. This approach resulted in a protection against diabetes-accelerated experimental OA in db/db mice. However, the numerous side effects associated to Rapamycin (Sirolimus) compromise its potential use as a preventive or therapeutic option. Currently, it is only approved by the European Medicines Agency (EMA) as an immunosuppressant drug for kidney transplantation. However, effective pharmacological mTOR inhibition may also be useful in other conditions. In order to avoid its side-effects, rapamycin analogues with improved pharmacokinetic properties, known as rapalogs, have been developed. However, despite presenting a better pharmacokinetic profile, similar problems are likely to emerge with rapalogs, likely limiting their future use (Benjamin et al, 2011; Lamming et al, 2013). Testing different dosing regimens or different administration routes could be a promising strategy to address this issue. In fact, for OA treatment, recent studies suggest the intra-articular administration of Rapamycin as a way to avoid systemic side effects. While the intra-articular administration of Rapamycin seems to maintain its protective effects, its ability to limit systemic side-effects was not yet thoroughly evaluated (Matsuzaki et al, 2014; Takayama et al, 2014). Thus, more studies, specifically focused on side effects, need to be performed. Moreover, further pharmacokinetic studies with mTOR inhibitors are needed to determine minimum effective doses and the right duration of exposure to induce autophagy with minimal side effects.

The acknowledgment that mTOR inhibition protects against cartilage degradation in diabetic conditions will stimulate research on many other compounds known to reduce mTOR activity. Besides Rapamycin there are other options to inhibit mTOR activity, namely adenosine triphosphate (ATP)-competitive mTOR inhibitors which inhibit both mTORC1 and mTORC2 (Torin-1), some of them with dual mTOR/PI3K inhibitory effect (PI-103) (Benjamin et al, 2011; Kim & Guan, 2015). In fact, a recent study demonstrated that an intra-articular injection of Torin-1, in a rabbit model of OA, established by intra-articular injection of collagenase, decreased the degenerative changes of knee joints (Cheng et al, 2016). As described above, targeting AMPK, an upstream negative mTOR regulator, is another possible route for mTOR inhibition. Metformin, the most widely prescribed antidiabetic drug, is an AMPK activator known to inhibit mTOR and consequently to induce autophagy (Meng et al, 2015; Zhou et al, 2001). Moreover, metformin also inhibits mTOR through an AMPK independent manner via Rag GTPase or REDD1 (Ben Sahra et al, 2011; Kalender et al, 2010). As a safe and well tolerated drug, investigating metformin as a therapeutic option to stop rapid progression of OA in diabetes should be considered. Glucosamine, as discussed earlier, is another drug with a known effect as a mTOR inhibitor but its clinical use in diabetic patients may be hampered by its complex interaction with insulin function (Monauni et al, 2000; Pham et al, 2007), as well as by its unfavorable pharmacokinetics (du Souich, 2014).

In fact, the possibility of multiple effects of the therapeutic options should also be considered. In this sense, it would also be interesting to fully characterize the effects on joint health of currently used interventions designed to control diabetes. In bone, different antidiabetic drugs seem to have beneficial or detrimental effects (Yan & Li, 2013). Regarding articular cartilage, some studies suggest a protective effect using anti-diabetic drugs. For instance, pioglitazone, a PPARγ agonist, reduces OA mediators and promotes anti-inflammatory responses (Boileau et al, 2007; Kobayashi et al, 2005). Moreover, glibenclamide, another antidiabetic drug currently used, was found to decrease the protein levels of GLUT-1 and -3 in normal or early OA stage human chondrocytes, but not in late OA stages. Whether this effect can prevent the intracellular glucose accumulation and its consequences in diabetes remains to be elucidated (Rufino et al, 2013). Further studies evaluating the impact of these antidiabetic drugs in chondrocytes, articular cartilage and in other joint tissues and also in *in vivo* models of diabetes-associated OA may also be required. Moreover, the impact of these drugs on autophagy modulation and possible counteraction of accelerated OA in diabetic patients is yet to be established. As stated previously, the one exception is metformin which is known to induce autophagy. However, its effects on cartilage integrity are still unknown, an observation that would be interesting to determine. Considering the role of metformin as an autophagy inducer, the possible protective effect of pioglitazone in articular cartilage, and their

current use in diabetic patients, a decrease in OA progression should be expected in patients treated with those drugs. In this sense, epidemiologic studies evaluating the impact of the anti-diabetic drugs in OA prevalence are also needed.

Beyond autophagy activation through mTOR inhibition, it would also be interesting to evaluate if pharmacologic strategies that induce autophagy through mTOR independent pathways have similar beneficial effects. This would help assert if the protective effect observed is exclusively linked to mTOR inhibition or due to direct autophagy activation. For instance, some compounds have been identified as autophagy modulators that act on targets like the imidazoline receptor (clonidine, rilmenidine) and L-type Ca 2+ channels (verapamil). Moreover, lithium, a nonspecific inhibitor of inositol monophosphatase, lowers inositol levels and consequently increases autophagy. B cell lymphoma 2 (Bcl-2) homology 3 (BH3) mimetics counteract the binding of Bcl-2 to Beclin-1, therefore also operating as autophagy inducers (Rubinsztein et al, 2012). Many of these drugs are currently used in clinical practice to control other conditions such as hypertension, thus presenting possible therapeutics options.

In conclusion, the results presented firmly support the existence of a diabetes-associated OA phenotype and identify, for the first time, autophagy impairment in chondrocytes and in articular cartilage under diabetic conditions. This may establishes a link between diabetes and OA, advancing our understanding of the mechanisms underlying this association. Furthermore, this also introduces pharmacological modulation of autophagy, through mTOR signaling, but possibly through other mechanisms, as a potential therapeutic target to prevent OA in this subset of patients. We hope that our results can help pave the way for the development of target-specific therapies that can halt OA progression in diabetic patients.

CHAPTER 5

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