UNIVERSIDADE DA CORUÑA
Facultade de Ciencias

Grao en Bioloxía

Memoria do Traballo de Fin de Grao

Metabolic flexibility in human chondrocytes, preliminary study.

Flexibilidade metabólica en condrocitos humanos, estudo preliminar.

Flexibilidad metabólica en condrocitos humanos, estudio preliminar.

Manuel Serna Vila

Curso: 2022- 2023. Convocatoria: Xullo

Directora 1: *Mercedes Fernández Moreno*
Directora 2: *Esther Rodríguez Belmonte*

ACKNOWLEDGMENTS:

I would like to express my gratitude to my mentors, Dr. Mercedes Fernández Moreno and Dr. Esther Rodríguez Belmonte, for allowing me to participate in this fascinating project. Notably, I am grateful to Mercedes for her assistance, support, and for her continuous guidance.

I would like to thank the INIBIC and CICA teams for their help in the laboratory and for their advice.

Lastly, I want to express my appreciation to my family and friends for their unconditional support during this academic journey.

INDEX:

ABSTRACT:

Osteoarthritis (OA) is the most common rheumatic condition affecting joint tissues and is characterized by molecular and metabolic impairments. Chondrocytes, the only cell type in the cartilage, primarily rely on glucose metabolism to generate energy. Maintaining metabolic flexibility, the ability to adapt to changing demands, is crucial for sustaining a healthy joint.

This study aimed to analyze the metabolic flexibility in primary human chondrocytes by analyzing the gene expression of several genes implicated in the glucose and fatty acid (FA) metabolic pathways. Chondrocytes were cultured in presence of glucose in combination with oleic acid. The glucose and FA metabolic pathways were inhibited using UK599 or Etomoxir respectively. Results showed that healthy (N) chondrocytes exhibited enhanced pyruvate transport to mitochondria and better FA metabolism compared with OA chondrocytes.

RESUMO:

A artrose (OA) é unha patoloxia crónica que afecta os tecidos articulares e caracterízase por alteracións moleculares e metabólicas. Os condrocitos, o único tipo celular presente na cartilaxe, dependen principalmente do metabolismo da glicosa para xerar enerxía. Manter unha flexibilidade metabólica, a capacidade de adaptarse ás demandas metabólicas cambiantes, é crucial para manter unha articulación saudable.

Este estudo tivo como obxectivo analizar a flexibilidade metabólica en condrocitos humanos. Para desenrrolar dito obxectivo as células creceronse en presenza de glicosa e ácido oleico e, cando foi preciso, a via metabolica de glicosa ou dos acidos grasos (FA) inhibiouse usando UK5099 ou Etomoxir en cada caso. Os resultados mostraron que os condrocitos sanos (N) presentaban un transporte de piruvato mais eficaz ás mitocondrias e un metabolismo de FA máis eficiente en comparación cos condrocitos afectados pola OA.

RESUMEN:

La artrosis (OA) es una patología crónica que afecta a todos los tejidos articulares y se caracteriza por alteraciones moleculares y/o metabólicas. Los condrocitos, único tipo celular presente en el cartílago, dependen principalmente del metabolismo de la glucosa para generar energía. Mantener una flexibilidad metabólica, la capacidad de adaptarse a las demandas cambiantes, es crucial para mantener una articulación saludable.

Este estudio tuvo como objetivo analizar la flexibilidad metabólica en condrocitos humanos. Para ello los condrocitos de crecieron en presencia de glucosa y en combinación con ácido oleico, y cuando fue necesario inhibir uno u otro metabolismo se utilizaron inhibidores como UK5099 o Etomoxir, respectivamente. Los resultados mostraron que los condrocitos sanos (N) presentaban un transporte de piruvato a las mitocondrias más eficaz y un metabolismo de ácidos grasos (FA) más eficiente en comparación con los condrocitos OA.

KEYWORDS:

Osteoarthritis, primary human chondrocytes, metabolic flexibility, gene expression.

ABBREVIATIONS AND SYMBOLS:

- ATP: Adenosine Triphosphate
- cDNA: Complementary DNA
- CTAB: Cetyltrimethylammonium Bromide
- DMEM: Dulbecco's Modified Eagle's Medium
- DNA: Deoxyribonucleic Acid
- EDTA: Ethylenediaminetetraacetic Acid
- FAs: Fatty Acids
- FBS: Fetal Bovine Serum
- H&E: Hematoxylin-eosin
- h: Hours
- min: Minutes
- mL: Milliliters
- M: Molar
- mM: Millimolar
- mtDNA: Mitochondrial DNA
- MW6: 6-Well Multiwell Plate
- N: Healthy
- ng: Nanograms
- nm: Nanometers
- OA: Osteoarthritic
- OV: Overnight
- OXPHOS: Oxidative phosphorylation
- PCR: Polymerase Chain Reaction
- (P/S): Penicillin and Streptomycin
- qRT PCR: Quantitative real-time PCR
- RNase H: Ribonuclease H
- RNA: Ribonucleic Acid
- ROS: Reactive Oxygen Species
- rpm: Revolutions per Minute
- RT: Room Temperature
- SBE: Single Base Extension
- SFG: Safranin O-Fast Green
- SNPs: Single-Nucleotide Polymorphisms
- TB: Toluidine Blue
- Tm: Melting temperature
- µl: Microliter
- µM: Micromolar
- µm: Micrometres
- ºC: Degrees Celsius

INTRODUCTION

1. INTRODUCTION

Osteoarthritis (OA) is a chronic progressive disorder that affects joint tissues. It is characterized by molecular alterations that lead to cellular stress, extracellular matrix degradation, and ineffective repair responses that promote inflammatory pathways along with metabolic processes, resulting in structural failure of the synovial joint (1).

Traditionally, OA was considered a cartilage-deficient condition. However, recent works have demonstrated that it is a disease of joint tissue as a whole, rather than an isolated cartilage disease (2). Studying OA requires the consideration of all associated processes and interconnected structures including cartilage degradation, subchondral bone remodeling, osteophyte formation, synovial membrane inflammation, and involvement of supporting tissues including ligaments, tendons, and muscles (3, 4) (**Figure 1**).

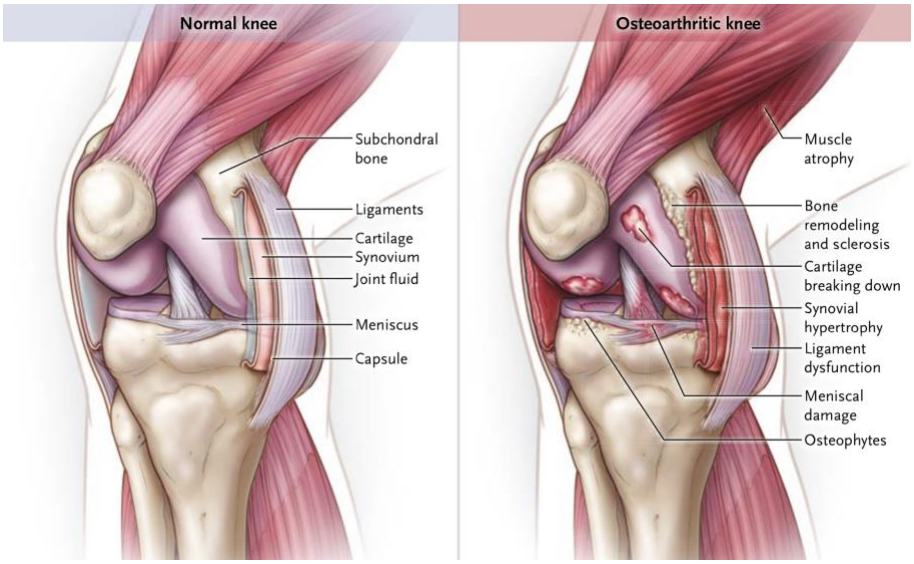


Figure 1: Comparison of a healthy (N) and an OA knee joint. The illustration above highlights how OA affects each component of the knee joint. Image extracted from Hunter D. 2015 (4).

OA imposes a substantial and growing burden on healthcare systems, with an average annual direct global medical cost per patient of $13,600 (5). It has become one of the most prevalent diseases among populations in high-income countries and with a rising trend (6). According to a study issued in 2020, the prevalence of OA in Spain was found to be 29.35% in one or more joint locations (7). Hip and knee OA contributes significantly to the overall cases, often leading to joint failure and the need for surgical replacement (6).

OA is a heterogeneous disease, and in recent years, researchers have identified various phenotypes and endotypes to better understand it. Defining these phenotypes is important; First, it helps us understand what factors contribute to different subtypes of OA from an epidemiological perspective. Second, it allows us to target treatments specifically for each phenotype, which can lead to more successful clinical trials (8, 9). There are six main phenotypes: chronic pain, inflammation, local metabolic changes in the joint, metabolic syndrome, mechanical overload, and minimal joint disease (8, 10).

Some of the factors that contribute to the OA phenotypes include age, ethnicity, gender, and genetics. Aging is linked to a higher risk of OA, also variations in OA incidence are observed among different racial and ethnic groups. Women have a higher incidence of knee OA, possibly influenced by hormonal factors related to menopause. Other risk factors involve obesity, previous knee injury, and some professional activities, which affect joint loading and biomechanics. Additionally, malalignment and anatomical abnormalities contribute to an increased risk of OA (11). Most of these factors result in metabolic impairment disrupting the homeostasis of chondrocytes (1, 12, 13).

The chondrocyte is the only cell type present in articular cartilage. Chondrocytes are responsible to produce and maintain the cartilage extracellular matrix, which is formed by collagen type II fibers and proteoglycans. This cell type plays an essential role in the equilibrium between anabolic and catabolic processes, maintaining the homeostasis of the cartilage (14). Numerous procedures, including nutrition sensing, signaling, transporting, storage, and oxidation, are involved in maintaining homeostasis. Under stress, cells can change how they use energy by either increasing catabolic pathways when there are substrates available or increasing anabolic activities when there is a lack of nutrients (15).

Chondrocytes in hyaline cartilage rely on diffusion through the extracellular matrix to obtain nutrients and oxygen as this type of tissue lacks nervous, blood vessels, and lymphatic systems (12, 16). Glucose is the main fuel and structural building compound in used by the chondrocytes, providing over 75% of cellular Adenosine Triphosphate (ATP) through glycolysis, while the remaining ATP (25 %) is produced through mitochondrial respiration by oxidative phosphorylation (OXPHOS) (17, 18).

Chondrocytes transport glucose into the cell using glucose transporters, mainly *SLC2A1* and *SLC2A3*. *SLC2A1* is upregulated in response to low oxygen and glucose levels but downregulated in high glucose environments, enabling efficient glucose uptake under low oxygen conditions, and maintaining glucose balance in cells. The expression of *SLC2A3* remains unchanged regardless of anabolic or catabolic stimuli (17). Therefore, under conditions of limited oxygen and nutrient availability, chondrocytes primarily rely on the glycolytic pathway for energy production.

Several studies indicated that free fatty acids (FAs) may play a significant role in the development of OA. The accumulation of FAs increases as OA progresses, leading to lipotoxicity and cellular dysfunction (19). The main FAs found in cartilage are oleic, palmitic, and linoleic acid, which make up around 85% of the total FAs content.

Metabolic flexibility is the ability to respond and adapt to changes in metabolic demand (20). Metabolic flexibility in skeletal muscle is well studied, it is regarded as a healthy trait and is directly correlated with healthy mitochondria, which are essential in glucose and fatty acid metabolism. On the other hand, metabolic rigidity results in impaired glucose or fatty acid oxidation, which causes nutrients accumulation (19).

Mitochondrial dysfunction, observed in human OA chondrocytes, has been associated with several pathways related to cartilage degradation, such as oxidative

stress, inflammation, matrix calcification of cartilage, and chondrocyte cell death (19, 21-23). Apoptosis and impaired autophagy are also crucial processes in the initiation and progression of OA (24, 25).

In addition to this, some continent-specific mtDNA polymorphisms, commonly referred as mtDNA haplogroups, modulate mitochondrial function influencing the metabolism of the cell and the progression of OA (26-29). The genetic composition of the European population is primarily represented by a set of nine major haplogroups, which include H, I, J, K, T, U, V, W, or X (30). Studies revealed a higher incidence of OA in patients with mtDNA haplogroup H, while haplogroups J and T were linked to a reduced incidence of OA (26-28, 31, 32).

Therefore, understanding the metabolic flexibility and their relationship with mitochondria alteration in chondrocytes is crucial for advancing in the knowledge of OA. This study aims to explore this aspect and provide insights into how metabolic changes contribute to the development of OA.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

Considering data described before, N chondrocytes possess a higher adaptive capacity than OA chondrocytes and, therefore, a higher metabolic flexibility.

To analyze metabolic flexibility in primary human chondrocytes obtained from healthy (N) and OA donors, we evaluated their different behaviors under metabolic stress conditions associated with OA.

To obtain this primary objective, we established two sub-objectives:

1.1 Stablish the Mankin score and mtDNA haplogroups.

1.2 Study the response of primary human chondrocytes culture in different conditions, analyzing the phenotype and the gene expression of several genes related with glucose and FA metabolism.

MATERIALS AND METHODS

3. MATERIALS AND METHODS:

3.1 SAMPLE DESCRIPTION:

3.1.1 Tissue obtained:

Human samples were recruited from the Rheumatology service area of the Hospital Universitario de A Coruña. Cartilage from hip replacement from healthy (N) and osteoarthritic (OA) donors was obtained from samples included in the Sample Collection for Research on Rheumatic Disease initiated by Dr. Francisco Blanco-García. This collection was approved by the Galician Research Ethics Committee and registered in the National Registry of Biobanks with registry code 2013/107 and Collections Section code C.0000424.

3.1.2 Chondrocyte isolation:

Cartilage dissection:

The aseptic dissection of articular cartilage from the hip joint was developed using a sterile scalpel. Cartilage slices were extracted and placed in a physiological saline solution and dissected into small parts.

Isolation of human chondrocytes:

The isolation of chondrocytes was performed through sequential enzymatic digestion. The cartilage was incubated in 10 mL of medium containing 1x Trypsin (Sigma-Aldrich, San Luis, USA) at 37ºC for 10 minutes (min) under agitation. Following, the supernatant was removed, and an extraction medium containing collagenase (2 mg/mL) (Sigma-Aldrich) was added and incubated overnight (OV) at 37º C. After incubation, the isolated cells were transferred to a 50 mL tube, and centrifugated at 1500 revolutions per minute (rpm) for 10 min, the supernatant was discarded. The chondrocytes were resuspended in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Paisley, UK) with Fetal Bovine Serum (FBS) (10%) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), penicillin (1000 µL/mL) (Invitrogen) and streptomycin (100 µL/mL) (Invitrogen) (P/S).

Cell expansion:

Cells were cultured in a flask (Corning Incorporated, Corning, NY, USA) and placed in an incubator with an atmosphere of 5% CO_2, 37ºC, and 95% humidity. The culture medium was replenished every 3-4 days until the cells reached almost 80% of confluence.

3.1.3 Cell culture:

To develop the experiments, the chondrocytes were cultured in $1x10^5$ cells/well in a multiwell plate with 6 wells (MW6) in DMEM with high glucose (4,5 g/L), FBS (10%) and (P/S).

The chondrocytes were cultured for the first 24 hours (h) in DMEM with 4.5 gr/L glucose, then glucose concentration was decreased to 1 gr/L. 24h after that, 100 µM of oleic acid (Sigma-Aldrich) was added and incubated for 24 h. The inhibitor UK5099, that

inhibits the *MPC* family, which are mitochondrial proteins involved in pyruvate metabolism (33), was added at 10 µM (Sigma-Aldrich) and Etomoxir inhibitor of *CPT1*, a gene that mediates in the transport of long-chain fatty acids by binding to carnitine, affecting the oxidation of fatty acids (34) was using at 20 µM (Sigma-Aldrich), both were added during 4h. When the incubation time finished, cells were processed for RNA extraction.

3.2 NUCLEIC ACID EXTRACTION AND QUANTIFICATION:

3.2.1 DNA extraction:

DNA isolation was developed using cartilage. A portion of 50 mg of each sample was digested in the presence of 500 µL of Cetrimonium Bromide (CTAB) buffer (2% CTAB, 100mM Tris-HCl (pH 8.0), 20 mM EDTA·Na_2·$2H_2O$, 1.4 M NaCl (Sigma-Aldrich)). First, a mechanical digestion using Noyes scissors was developed and then an enzymatic digestion was performed using 5 µL of Proteinase K (Qiagen, Hilden, Germany), incubated OV at 60ºC with shake.

Once the incubation was finished, 2 µL of RNase H (Qiagen) were added and incubated at 37°C for 20 min. After, 500 µL of Chloroform (Sigma-Aldrich) was added, incubated for 5 min and centrifugated at 4ºC, at 12000 rpm for 10 min. The supernatant was mixed with 300 µL of isopropanol (Sigma-Aldrich) and kept at -20ºC OV. After, centrifugation was performed at 4 ºC, at 12000 rpm for 30 min. The pellet was washed with 1 mL of 70% cold ethanol (Supelco, Sigma-Aldrich). Finally, a centrifugation at 4 ºC, at 12000 rpm for 30 min was developed, and DNA obtained was resuspended in sterile water.

3.2.2 RNA extraction:

Cells were treated with 700 µL TRIzol™ Reagent (Invitrogen) for RNA isolation. Then, 300 µL of chloroform was added and mixed by inversion, and incubated at room temperature (RT) for 2 min. Once the incubation was finished, the samples were centrifuged at 4 ºC, at 12000 rpm for 15 min. During this centrifugation process, the homogenate was separated into a clear upper aqueous layer (containing RNA), an interphase and an organic layer (containing DNA and proteins). RNA was precipitated from the aqueous phase with 700 µL of isopropanol (Sigma-Aldrich) and 1 µL of glycogen (Sigma-Aldrich), allowing a 10 min incubation time at RT, samples were centrifuged at 4 ºC, at 12000 rpm for 10 min.

The supernatant was discarded and 1 mL of cold ethanol at 70% was added and then the pellet was resuspended centrifuging the samples at 4 ºC, at 12000 rpm for 5 min. Finally, the pellet was resuspended in 15 µL of Rnase-free water.

3.2.3 Quantification:

Nanodrop-NA-100 spectrophotometer (Thermo Fisher) was used for the DNA and RNA quantification. It was useful for determining the concentration of each sample (expressed in ng/µL), also its purity and integrity.

The quantification wavelength was 260 and 280 nanometers (nm). The 260/280 ratio determines integrity. A score of ≤ 1.5 denotes contamination with proteins and/or cellular debris. The ratio of 260/230 was used to measure each sample purity, contamination with phenolic compounds was indicated by a value of ≤ 1.5.

3.3 MITOCHONDRIAL DNA (mtDNA) HAPLOGROUP DETERMINATION:

mtDNA haplogroups were assigned following the methodology described by Rego *et al.*, 2008 (26). Multiplex-PCRs were performed to amplify mtDNA fragments that contained the informative single nucleotide polymorphisms (SNPs) that characterized the most common Caucasian mtDNA haplogroups (H, J, K, T, and U). The resulting PCR fragments were analyzed by single base extension (SBE). The SBE was loaded into an ABI 3130XL genetic analyzer (Applied Biosystems, Thermo Fisher Scientific).

3.4 QUANTITATIVE REAL-TIME PCR (qRT PCR):

3.4.1 RNA reverse transcription:

RNA was retrotranscribed to complementary DNA (cDNA) using the NZY First-Strand cDNA Synthesis Kit (Nzytech, Lisbon, Portugal). 500 ng of RNA were retrotranscribed from each sample; 10 µL NZYRT 2× Master Mix and 2 µL of NZYRT Enzyme Mix were added, then mixed gently and incubated at 25 °C for 10 min, followed by 50 ºC for 30 min and 85 °C for 5 min. Once this process finished 1 µL of NZY RNase H was added and incubated at 37 °C for 20 min. The cDNA obtained was diluted in a 1:10 ratio in ultrapure H_2O and stored at RT.

3.4.2 qRT PCR:

qRT PCR analysis was developed in the LightCycler® 480 II instrument (Roche, Basel, Switzerland). SYBR Green I was employed to measure the generation of PCR products. SYBR Green intercalates with the DNA helix, resulting in an increase in fluorescence upon DNA binding. Thus, the amount of double-stranded DNA produced during PCR was directly proportional to the fluorescence intensity of SYBR Green.

To ensure the specificity of the PCR product, a melting curve analysis was performed to verify the presence of a single specific product peak. The melting temperature (Tm) refers to the temperature at which half of the double-stranded DNA transforms into single-stranded DNA.

The reaction mixture included: 10 µL of SYBR Green (Roche), 0.3 µL of each gene (20 µM), 3,5 µL of cDNA, and water to obtain a final volume of 20 µL. The amplification process using the LightCycler® 480 II instrument (Roche) was described in **Appendix-Ⅰ.** The primers sequences used for the gene amplification were listed in **Table 1** and the gene function was depicted in **Table 2.**

The gene expression analysis was conducted using Qbase plus version 2.5 software (Biogazelle, Ghent, Belgium). Gene expression levels were calculated relative to the housekeeping genes Glyceraldehyde 3-phosphate Dehydrogenase (*GAPDH*) and Ribosomal Protein L13a (*RPL13A*).

TABLE 1: Primers sequences used in the experiments.

Gene name	Primer forward 5'-3'	Primer reverse 3'-5'
MPC1	GGAAGACCCCAGTTGGCTAC	AGGATTTCCGGGACTACCTC
MPC2	TGGAGCCCAGAAGAAAACTG	ATGCTGCCCAGAAAATTG
CPT1	GAGCAGCACCCCAATCAC	AACTCCATAGCCATCATCTGCT
FASN	TTCTGGGACAACCTCATCG	AGACAGGTCCTTCAGCTTGC
SLC2A1	AGTGTCACCCACAGCCTTTC	AGTCCAGGCCGAACACCT
SLC2A3	GGGTGTGGTTAATACTATCTTCACTG	TCATATGCAGAGTCCTTCTTCCT
HK2	AGAAGCTCCCACTGGGTTTT	CATGAGACCAGGAAACTCTCG
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
RPL13A	CAAGCGGATGAACACCAAC	TGTGGGGCAGCATACCTC

TABLE 2: List of genes analyzed in this study, illustrated by their name and function.

Genes	Long name	Function
MPC1 and MPC2	Mitochondrial Pyruvate Carrier 1 and Mitochondrial Pyruvate Carrier 2	The proteins encoded by these genes are part of an MPC1/MPC2 heterodimer that is responsible for transporting pyruvate into mitochondria.
CPT1A	Carnitine Palmitoyltransferase 1A	This gene is responsible for initiating the mitochondrial oxidation of long-chain fatty acids by facilitating the transport of these fatty acids across the mitochondrial inner membrane.
FASN	Fatty Acid Synthase	This gene encodes a multifunctional enzyme that synthesizes saturated fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH.
SLC2A1	Solute Carrier Family 2 Member 1	Also known as Glut-1, is the most important glucose transporter in mammals, it is expressed in chondrocytes.
SLC2A3	Solute Carrier Family 2 Member 3	This gene encodes a protein known as Glut-3, another glucose transporter present in chondrocytes.
HK2	Hexokinase 2	HK2 encodes an enzyme that plays a crucial role in the initial step of glucose metabolism by generating glucose-6-phosphate through the addition of a phosphate group to glucose.

3.5 HISTOLOGICAL ANALYSIS:

Histological study was carried out to define the cartilage state. Hip joints from donors were harvested and fixed OV in 4% formaldehyde (Sigma-Aldrich) and then samples were paraffin embedding. Serial sections of 6 µm were obtained. All these procedures were developed by the Histomorphology Area from GIR-S in the CICA.

A deparaffinization was performed for 30 min at 60 ºC, then a 10 min Xylol (Sigma-Aldrich) immersion, after and hydration was developed in decreasing alcohols: 100º alcohol for 5 min then 96º alcohol for another 5 min and then rinsed in water.

For processing and studying the tissues, three different stainings were developed:

Hematoxylin-eosin (H&E) stain:

Immersion in Hematoxylin stain (Sigma-Aldrich) was conducted, then the sample was rinsed in hot water, and subsequently, a 5 min immersion in Eosin (0,07%) (Sigma-Aldrich) was conducted.

Toluidine blue (TB) stain:

Immersion in TB stain (Sigma-Aldrich) for 5 min was developed, then the sample was rinsed in an acetic acetate tampon, and subsequently, it was immersed in distilled water.

Safranin o-fast green (SFG) stain:

Immersion in Safranin-o (Sigma-Aldrich) for 30 min followed by an immersion in Fast green (Sigma-Aldrich) for 30 min. The samples were immersed in distilled water, then in 100º alcohol twice, and finally in Xylol (Sigma-Aldrich).

To finish all the staining, the samples were immersed in an increasing alcohol degree set (90º-96º-100º). The samples were mounted with DePex (Sigma-Aldrich). The slides were analyzed under optical microscopy (Leica Microsystems, Wetzlar, Germany).

The Mankin score was established by selecting three criteria from the histological assessment: structure, cellularity, and matrix staining. An average score was used in the analysis following the criteria established by Pascual Garrido *et al.*, 2009 (35) as shown in **Appendix-Ⅱ**.

3.6 STATISTICAL ANALYSIS:

Statistical analysis was performed with the GraphPad Prism version 8.0.0 for Windows, (GraphPad Software, San Diego, California, USA, www.graphpad.com). The data were analyzed by the Student t-test for two group comparisons. P value lower than 0.05 was considered significant. All the measurement data were presented as mean ± Standard error of the mean (SEM).

RESULTS

4. RESULTS:

4.1 MANKIN SCORE:

Mankin score was employed to assess the presence and severity of OA.

The analysis of the staining images corresponding to H&E, TB, and SFG from healthy (N) and osteoarthritic (OA) donors showed that the N reflected a consistent superficial layer, no cellular abnormalities, and a correct matrix staining while OA samples had an irregular surface, fissures into the deep zone, cellular abnormalities, and a reduced staining in SFG (**Figure 2**).

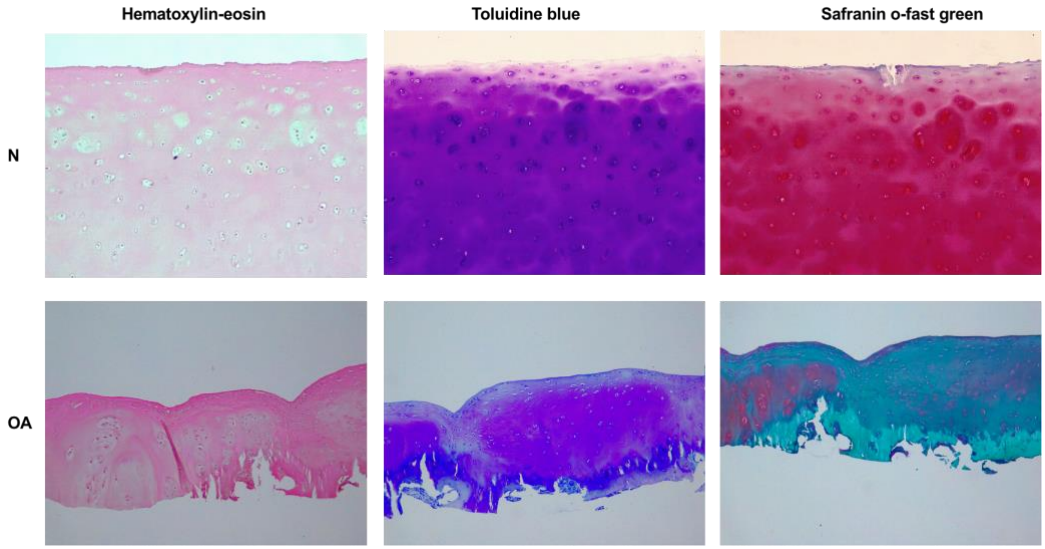


Figure 2: **An example of the H&E, TB and SFG staining in N and OA samples.** The first row corresponds to cartilage from N donor and the second row corresponds to OA donor, each column corresponds to a different stain (H&E, TB and SFG). Images were taken at 100 magnifications. N = Healthy donor and OA = Osteoarthritic donor.

When we analyzed the data corresponding to the Mankin score from all the samples used in this work, we observed significantly higher values in OA samples in comparison with N samples (**Figure 3**). This data confirmed the correct classification of the samples.

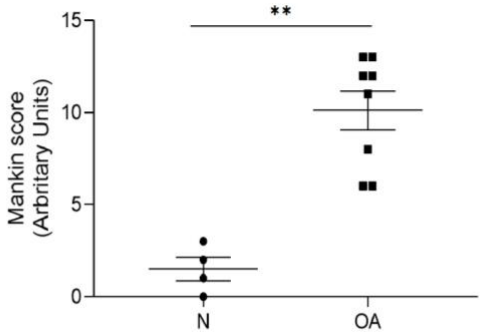
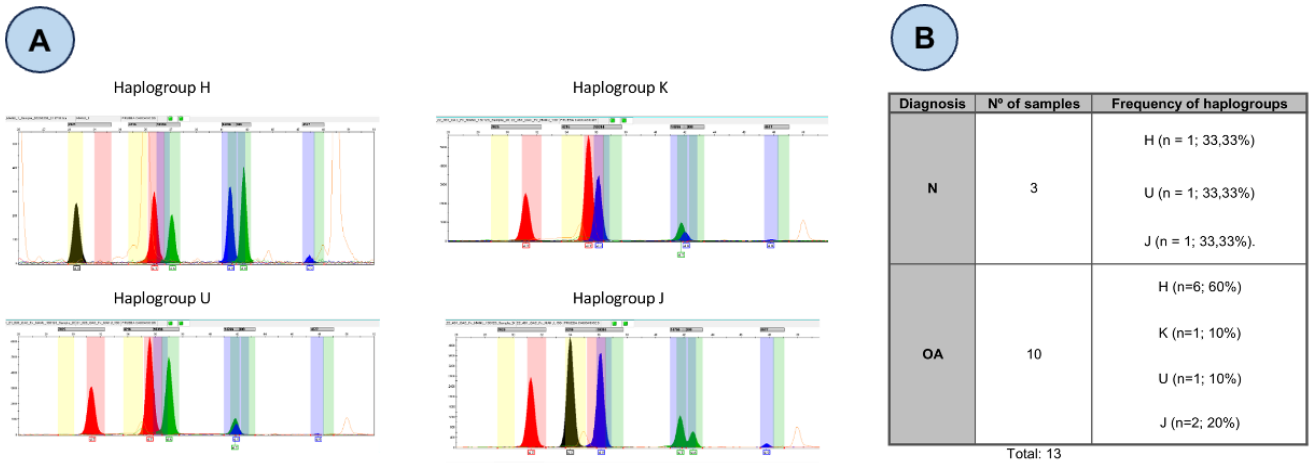


Figure 3: **Mankin score.** Statistical analysis of the Mankin score values among N and OA. All data were obtained from twelve independent donors (4 N and 8 OA). Values were presented as mean ± SEM and analyzed by t Student test (**p ≤ 0.005).

4.2 mtDNA HAPLOGROUP DETERMINATION:

mtDNA haplogroups were identified in 13 samples obtained from hip N and OA donors based on the combination of SNPs as shown in **Figure 4-A**.

The N samples analyzed in this study were obtained from 3 donors and each of them had different mtDNA haplogroup (H, U, and J). The OA samples analyzed were from 10 donors, the mtDNA haplogroup H, was detected in six samples and mtDNA haplogroups K, U, J, and T were present in the other four samples (**Figure 4-B)**.



Diagnosis	Nº of samples	Frequency of haplogroups
N	3	H (n = 1; 33,33%) U (n = 1; 33,33%) J (n = 1; 33,33%).
OA	10	H (n=6; 60%) K (n=1; 10%) U (n=1; 10%) J (n=2; 20%)

Total: 13

Figure 4: Haplogroup distribution among the different donors. A. SNPs panels used on the determination for each haplogroup. **B.** Representation of the distribution and frequency of each mtDNA haplogroup in all donors analyzed.

4.3 MORPHOLOGICAL CHANGES DURING CELL CULTURE:

The results corresponding to the phenotypic changes of the chondrocytes cultured under different conditions were analyzed (**Figure 5)**.

Chondrocytes cultured under standard conditions (glucose 4.5 gr/L) showed their typical morphology (**Figure 5-A**). However, when glucose was decreased (glucose 1 gr/L) and the oleic acid (100 µM) were added, the morphology of the cells changed, and lipid droplets appeared in the chondrocytes (**Figure 5-B**). When the inhibitors of glucose (UK5099 10 µM) or FA (Etomoxir 20 µM) transporters were added, the morphology of chondrocytes changed owing to an increase in cellular stress and with the increased of culture time the cells started to die (**Figure 5-C**).

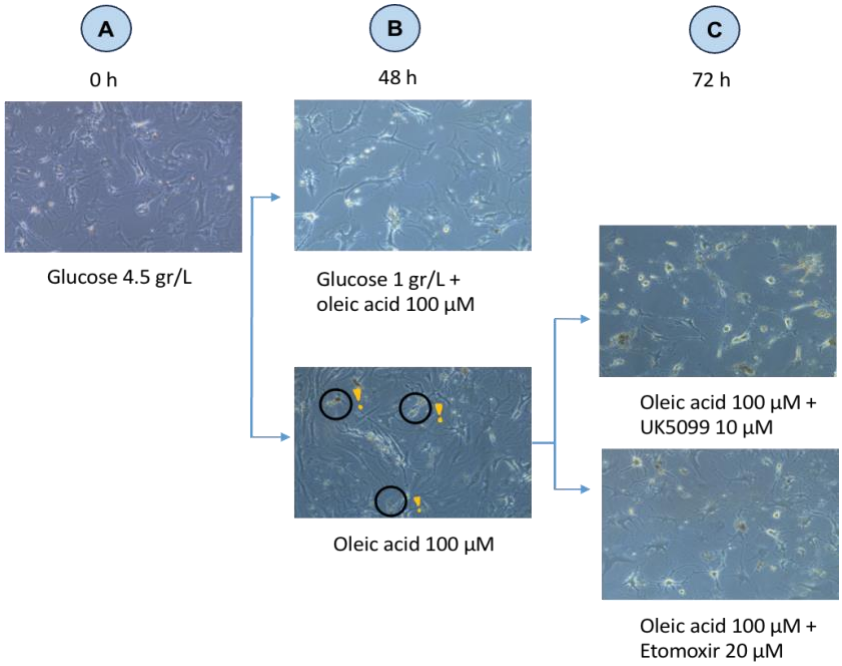
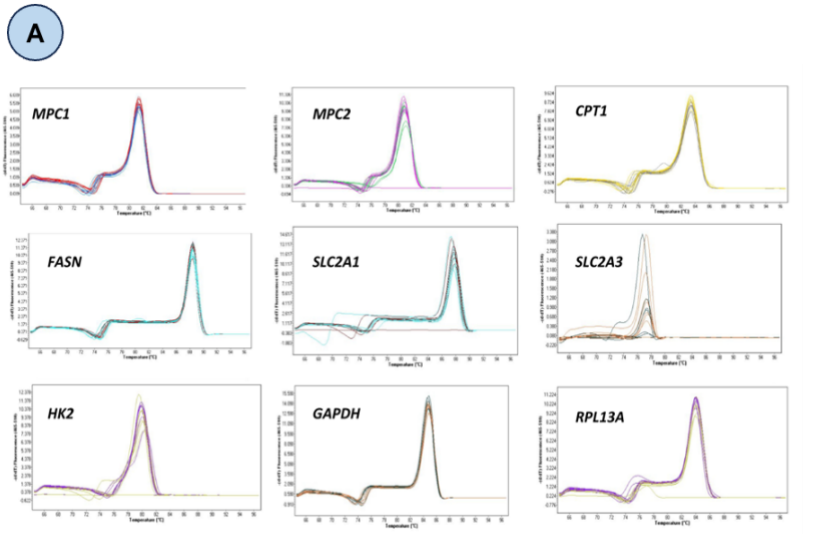


Figure 5: Chondrocyte growing using different culture mediums. **A.** cells cultured in DMEM with glucose 4.5 gr/L. **B.** 48 h since the beginning of the experiment the medium was changed to DMEM with 1 g/L glucose with the addition of 100 µM oleic acid. **C.** inhibitors were added to the culture: UK5099 (10 µM) and Etomoxir (20 µM). The experiment was concluded 4 h after the addition of the inhibitors. The yellow marks highlight the lipid droplets. The images illustrated are at 100 magnifications.

4.4 GENE EXPRESSION:

The relative gene expression levels of *MPC1*, *MPC2*, *CPT1A* (*CPT1*), *FASN*, *SLC2A1, SLC2A3* and *HK2* were analyzed. The melting curve of each gene was represented in **Figure 6-A** and the Tm values for each gene were described in **Figure 6-B**. Data showed that all the primers designed showed a~~n~~ unique amplification curve, with the exception of *SLC2A3*, for this reason, this gene was eliminated for the next experiment.



B

Genes	TMs
MPC1	81.4°C
MPC2	80°C
CPT1	83°C
FASN	88°C
SLC2A1	87°C
SLC2A3	77.2°C
HK2	79.5°C

Figure 6: Representation of the Tms of the different genes analyzed. A. Illustration of the melting curves. **B.** Numerical Tm value for each gene.

The first approach to relative gene expression was performed in the chondrocyte culture under basal condition. The data analysis showed that there was a lower gene expression tendency in OA for the *MPC1*, *MPC2,* and *CPT1* while *FASN* reflected a light increased expression in comparison with N (**Figure 7**).

The gene expression of genes related with glucose metabolism, *SLC2A1* and *HK2,* data showed that there was no difference between N and OA (**Figure 7**).

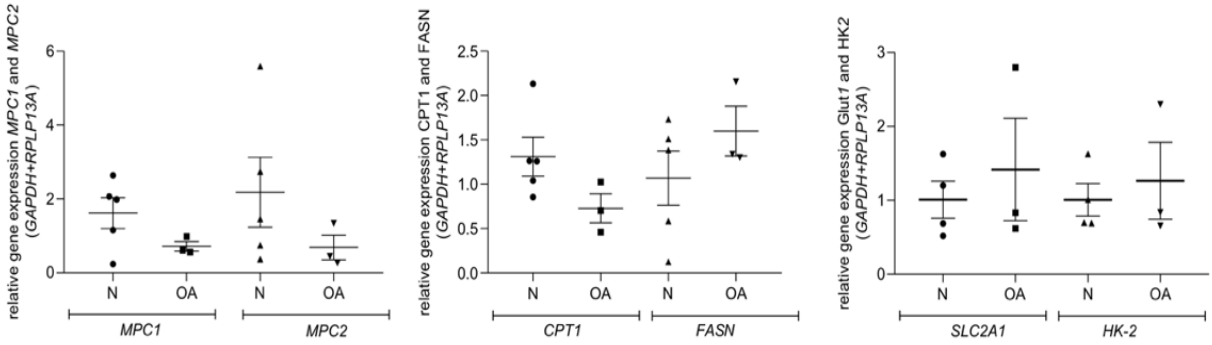


Figure 7: Relative gene expression under basal condition of six genes involved in glucose and FA metabolic pathways. *mitochondrial pyruvate carriers 1* and *2* (*MPC1* and *MPC2*), *carnitine palmitoyltransferase 1* (*CPT1*), *fatty acid synthase* (*FASN*), *glucose transporter 1* (*SLC2A1*) and *hexokinase-2* (*HK-2*). Data were obtained from eight independent donors (5 N and 3 OA) except for *SLC2A1* and *HK2* which they were obtained from 7 independent donors (4 N and 3 OA). Values were presented as mean ± SEM.

Based on these results, we decided to continue studying these genes but under different stimuli that modulate glucose and FA metabolism.

The gene expression of *MPC1* and *MPC2* in N samples showed a decrease when oleic acid was added. The incubation of N chondrocytes with UK5099 slightly increased the gene expression of *MPC1* and *MPC2* **(Figure 8-A)**. When the expression of both genes were evaluated in OA chondrocytes data showed a decreased when both culture conditions were compared. However, UK5099 generated a decrease of *MPC1* and *MPC2* gene expression **(Figure 8-B)**. The comparison between N and OA reflected a lower gene expression in the three conditions evaluated in OA chondrocytes **(Figure 8-C).** It was observed that N chondrocytes had a better performance in UK5099, when compared to OA chondrocytes.

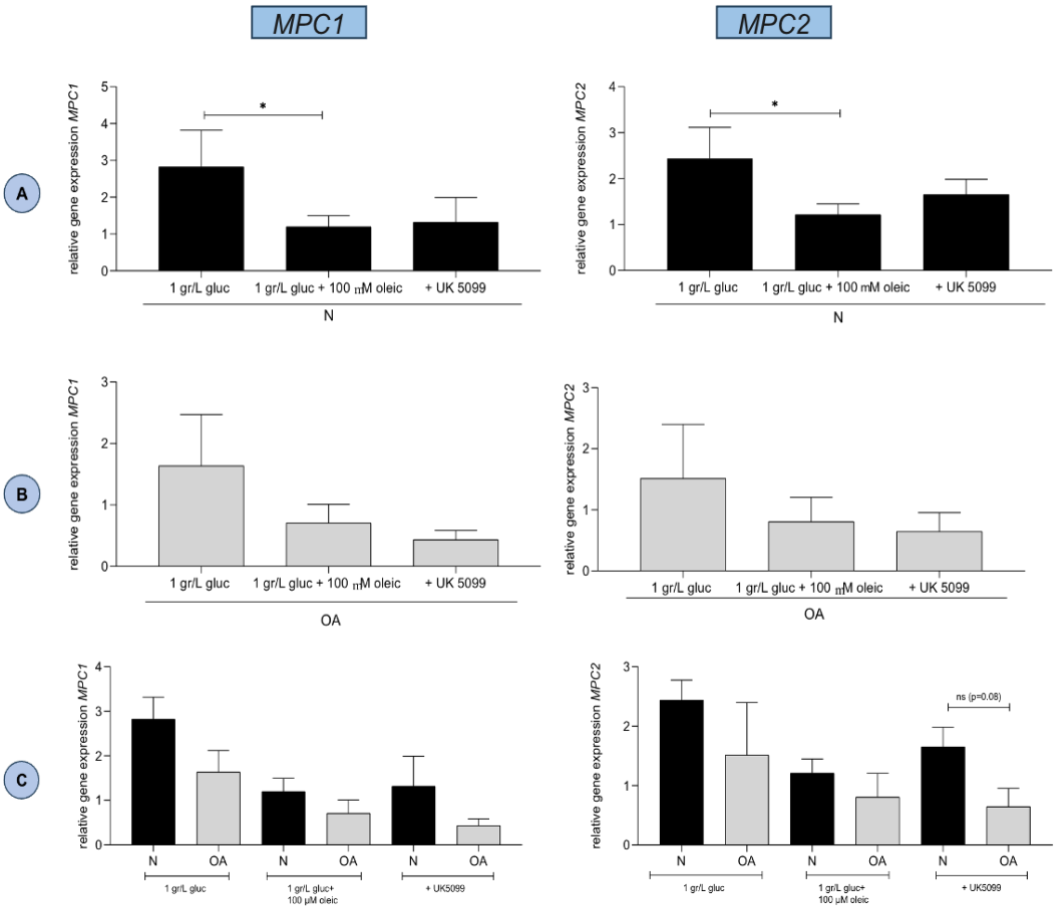


Figure 8: Relative gene expression of *MPC1* and *MPC2*. A. In N chondrocytes. **B**. In OA chondrocytes. **C**. Comparison between N and OA. Oleic Ac = Oleic acid, gluc = glucose, N = healthy chondrocytes, OA = Osteoarthric chondrocytes. This study was performed with a total of 7 samples (4 N and 3 OA), values were presented as mean ± SEM. Analyzed by t Student test (*p ≤ 0.005).

Relative gene expression of *CPT1*, gene related with FA metabolism, showed that N chondrocytes responded increasing its expression under the different conditions tested **(Figure 9-A)**. The analysis of OA reflected that the presence of oleic acid increased the *CPT1* gene expression, but when Etomoxir was present, the gene expression decreased **(Figure 9-B)**. The evaluation of *CPT1* modulation between N and OA showed that N chondrocytes had the capacity to increase the gene expression in oleic acid alone and in combination with Etomoxir. However, OA chondrocytes had a lower capacity of modulation **(Figure 9-C)**. These results demonstrated that N chondrocytes upregulated their expression of *CPT1* to facilitate the metabolism of fatty acids.

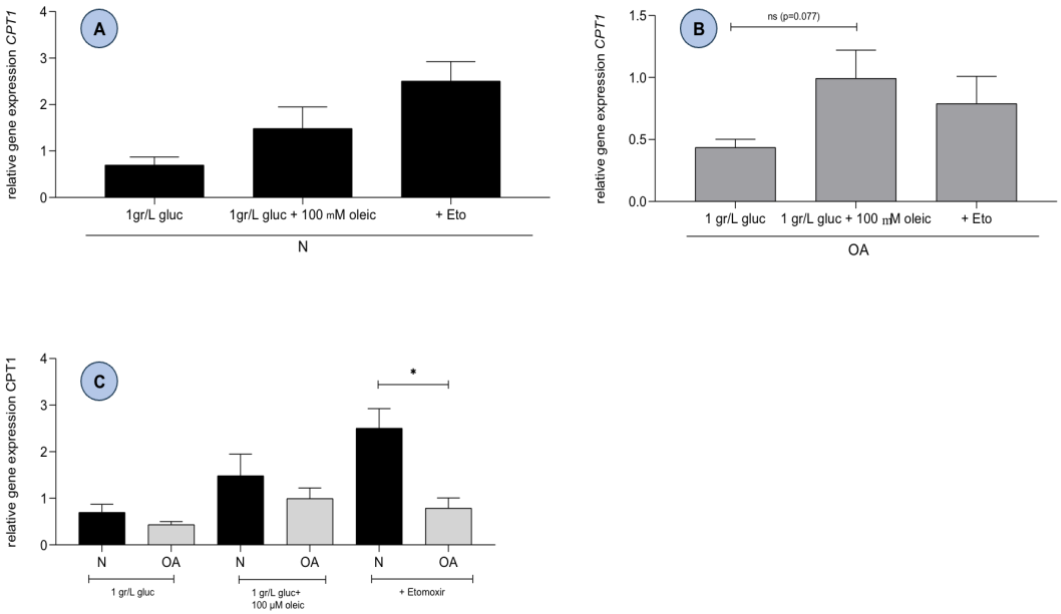


Figure 9: **mRNA expression of Carnitine palmitoyltransferase I (*CPT1*) normalized to the expression of *GAPDH*. A**. In N chondrocytes. **B**. In OA chondrocytes. **C**. Comparison between N and OA. Oleic Ac = Oleic acid, gluc = glucose, Eto = Etomoxir, N = healthy chondrocytes and OA = Osteoarthric chondrocytes. This study was performed with a total of 7 samples (4 N and 3 OA), values were presented as mean ± SEM. Analyzed by t Student test (*p ≤ 0.005).

Relative gene expression of *SLC2A1*, a gene involves in glucose metabolism, did not show variability among the stimuli in N and OA chondrocytes **(Figure 10-A-B)**. However, when comparing N and OA chondrocytes, a higher expression of *SLC2A1* was observed in OA chondrocytes **(Figure 10-C)**. These results showed that OA chondrocytes increased the expression of *SLC2A1* in order to capture more glucose.

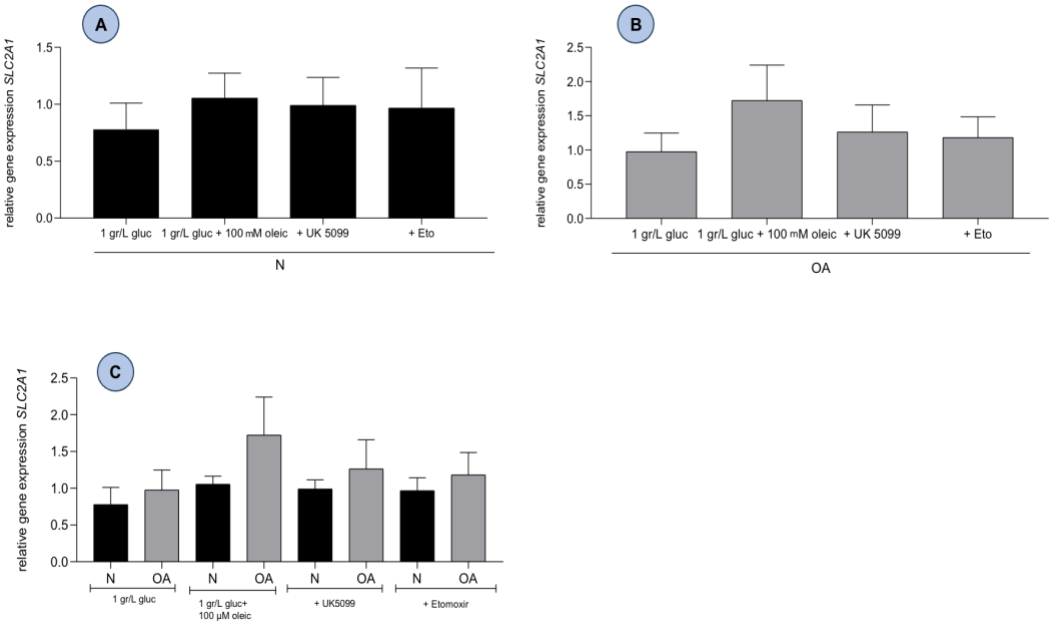


Figure 10: **Relative gene expression of *SLC2A1*. A**. In N chondrocytes. **B**. In OA chondrocytes. **C**. Comparison between N and OA. Oleic Ac = Oleic acid, gluc = glucose, Eto = Etomoxir, N = healthy chondrocytes and OA = Osteoarthric chondrocytes. This study was performed with a total of 7 samples (4 N and 3 OA), values were presented as mean ± SEM.

DISCUSSION

5. DISCUSSION:

Results of this study have contributed to the knowledge of the complex metabolic processes implicated in OA and the factors that may account for variations in metabolic flexibility among N and OA chondrocytes.

In recent years, there has been a growing interest in exploring the role of metabolic alterations in the development and progression of OA. Changes in gene expression related to metabolic pathways have been observed in OA chondrocytes, suggesting dysregulation of cellular metabolism (17, 19).

Chondrocytes rely primarily on glycolysis to meet cellular energy needs, but recent studies associate impaired mitochondrial function in OA pathogenesis (22). With this idea other authors investigated the ability of chondrocytes to upregulate mitochondrial respiration when challenged with a nutrient stress and determine the effect on mediators of chondrocyte oxidative homeostasis (19, 36). The ability to respond and adapt to changes in metabolic demand has been described as metabolic flexibility (37). Metabolic flexibility is associated with well-functioning mitochondria due to their role in the last steps of both glucose and FA metabolism (38).

To study if different substrates as glucose and oleic acid and some inhibitors could influence in the metabolism in N and OA cybrids, gene expression of genes related with glucose and fatty acid metabolism were examined.

OA chondrocytes primarily rely on glycolysis as their main energy source, but homeostatic and anti-inflammatory cells are characterized by implementing OXPHOS (41, 42). Metabolic alterations in the pyruvate pathway were observed in OA (39). For this, the pyruvate uptake from the cytoplasm to mitochondria was evaluated through the gene expression of *MPC1* and *MPC2.* Both genes generate a *MPC1*/*MPC2* heterodimer that is responsible for transporting pyruvate into mitochondria (40, 41). Results showed that when the oleic acid was present in the medium N chondrocytes decreased the gene expression of both genes and a light increase of both was described in presence of UK5099. Furthermore, OA chondrocytes displayed lower expression levels compared to N. The analysis of the *MPC* genes was included due its importance in the pyruvate metabolic pathway, linking glycolysis with OXPHOS, being a rate-limiting step in glycolysis (40).

Recent studies have described that N chondrocytes had a better metabolic flexibility when compared to OA chondrocytes (13, 19). In this study this fact was evaluated preliminarily analyzing the gene expression of *CPT1,* a mitochondrial carrier of FAs (34). In all conditions evaluated, the N chondrocytes showed higher gene expression of *CPT1* than OA. This suggested that N chondrocytes when oleic acid is present, have a better response capacity than OA chondrocytes. The analysis between N and OA gave us an idea that perhaps N chondrocytes were able to increase *CPT1* expression under stress condition in order to metabolize FA while OA chondrocytes had a lower incomplete FA oxidation as described in a cybrids study where the OA cybrids

were associated with lower gene expression of *CPT1B* and a higher lipid droplets accumulation than N cybrids (19).

A study based in transmitochondrial cybrids described that OA cybrids had a higher glucose uptake than N cybrids. In the literature, it was described that N chondrocytes can adjust to changes in glucose levels by controlling how much glucose they take in (15). They do this mainly by regulating the expression of *SLC2A1*, protecting chondrocytes from the harmful effects of having too much glucose build up inside them (13, 15, 42). In our study we observed a higher relative gene expression of *SLC2A1* in OA chondrocytes compared to N chondrocytes, particularly in the presence of oleic acid. This observation suggested that OA chondrocytes upregulate *SLC2A1* to enhance glucose uptake and meet their metabolic demands.

These preliminary data suggest that N and OA chondrocytes have a different capacity to respond under metabolic stress.

CONCLUSIONS

6. CONCLUSIONS:

With data obtained in this work we stablished the next conclusions:

1.1. Mankin score values confirmed the classification of samples, showed higher values in OA than in N samples

1.2. The distribution of mtDNA haplogroups in samples showed the normal frequency in European population where the H haplogroup is the most prevalent.

2.1. The nutrients disponibility affected to chondrocyte morphology increasing the lipid droplets and the death of cells.

2.2. Data revealed changes in gene expression related to metabolic pathways in OA chondrocytes under stressful conditions, suggesting that N chondrocytes have higher metabolic flexibility.

BIBLIOGRAPHY

7. BIBLIOGRAPHY:

1. Berenbaum F, Eymard F, Houard X. Osteoarthritis, inflammation and obesity. Curr Opin Rheumatol. 2013;25(1):114-8.
2. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum. 2012;64(6):1697-707.
3. Blanco FJ. Osteoarthritis and atherosclerosis in joint disease". Reumatol Clin (Engl Ed). 2018;14(5):251-3.
4. Hunter DJ. Viscosupplementation for osteoarthritis of the knee. N Engl J Med. 2015;372(11):1040-7.
5. Leifer VP, Katz JN, Losina E. The burden of OA-health services and economics. Osteoarthritis Cartilage. 2022;30(1):10-6.
6. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. Lancet. 2019;393(10182):1745-59.
7. Blanco FJ, Silva-Díaz M, Quevedo Vila V, Seoane-Mato D, Pérez Ruiz F, Juan-Mas A, et al. Prevalence of symptomatic osteoarthritis in Spain: EPISER2016 study. Reumatol Clin (Engl Ed). 2020.
8. Roemer FW, Jarraya M, Collins JE, Kwoh CK, Hayashi D, Hunter DJ, et al. Structural phenotypes of knee osteoarthritis: potential clinical and research relevance. Skeletal Radiol. 2022.
9. Mobasheri A, Saarakkala S, Finnilä M, Karsdal MA, Bay-Jensen AC, van Spil WE. Recent advances in understanding the phenotypes of osteoarthritis. F1000Res. 2019;8.
10. Van Spil WE, Kubassova O, Boesen M, Bay-Jensen AC, Mobasheri A. Osteoarthritis phenotypes and novel therapeutic targets. Biochem Pharmacol. 2019;165:41-8.
11. Chaganti RK, Lane NE. Risk factors for incident osteoarthritis of the hip and knee. Curr Rev Musculoskelet Med. 2011;4(3):99-104.
12. He Y, Li Z, Alexander PG, Ocasio-Nieves BD, Yocum L, Lin H, et al. Pathogenesis of Osteoarthritis: Risk Factors, Regulatory Pathways in Chondrocytes, and Experimental Models. Biology (Basel). 2020;9(8).
13. Zheng L, Zhang Z, Sheng P, Mobasheri A. The role of metabolism in chondrocyte dysfunction and the progression of osteoarthritis. Ageing Res Rev. 2021;66:101249.
14. Bai Y, Gong X, Dou C, Cao Z, Dong S. Redox control of chondrocyte differentiation and chondrogenesis. Free Radic Biol Med. 2019;132:83-9.
15. Mobasheri A, Rayman MP, Gualillo O, Sellam J, van der Kraan P, Fearon U. The role of metabolism in the pathogenesis of osteoarthritis. Nature reviews Rheumatology. 2017;13(5):302-11.
16. Guilak F, Nims RJ, Dicks A, Wu CL, Meulenbelt I. Osteoarthritis as a disease of the cartilage pericellular matrix. Matrix Biol. 2018;71-72:40-50.
17. Wu X, Fan X, Crawford R, Xiao Y, Prasadam I. The Metabolic Landscape in Osteoarthritis. Aging Dis. 2022;13(4):1166-82.
18. Blanco FJ, López-Armada MJ, Maneiro E. Mitochondrial dysfunction in osteoarthritis. Mitochondrion. 2004;4(5-6):715-28.
19. Dalmao-Fernández A, Lund J, Hermida-Gómez T, Vazquez-Mosquera ME, Rego-Pérez I, Blanco FJ, et al. Impaired Metabolic Flexibility in the Osteoarthritis Process: A Study on Transmitochondrial Cybrids. Cells. 2020;9(4).
20. Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. Am J Physiol Endocrinol Metab. 2008;295(5):E1009-17.
21. Hollander JM, Zeng L. The Emerging Role of Glucose Metabolism in Cartilage Development. Curr Osteoporos Rep. 2019;17(2):59-69.
22. Blanco FJ, Rego I, Ruiz-Romero C. The role of mitochondria in osteoarthritis. Nat Rev Rheumatol. 2011;7(3):161-9.
23. Vaamonde-García C, Riveiro-Naveira RR, Valcárcel-Ares MN, Hermida-Carballo L, Blanco FJ, López-Armada MJ. Mitochondrial dysfunction increases inflammatory responsiveness to cytokines in normal human chondrocytes. Arthritis Rheum. 2012;64(9):2927-36.
24. Wang H, Su J, Yu M, Xia Y, Wei Y. PGC-1α in osteoarthritic chondrocytes: From mechanism to target of action. Front Pharmacol. 2023;14:1169019.

25. Lotz MK, Caramés B. Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. Nat Rev Rheumatol. 2011;7(10):579-87.
26. Rego-Pérez I, Fernández-Moreno M, Fernández-López C, Arenas J, Blanco FJ. Mitochondrial DNA haplogroups: role in the prevalence and severity of knee osteoarthritis. Arthritis and rheumatism. 2008;58(8):2387-96.
27. Soto-Hermida A, Fernández-Moreno M, Pértega-Díaz S, Oreiro N, Fernández-López C, Blanco FJ, et al. Mitochondrial DNA haplogroups modulate the radiographic progression of Spanish patients with osteoarthritis. Rheumatology international. 2015;35(2):337-44.
28. Soto-Hermida A, Fernández-Moreno M, Oreiro N, Fernández-López C, Rego-Pérez I, Blanco FJ. mtDNA haplogroups and osteoarthritis in different geographic populations. Mitochondrion. 2014;15:18-23.
29. Shen JM, Feng L, Feng C. Role of mtDNA haplogroups in the prevalence of osteoarthritis in different geographic populations: a meta-analysis. PLoS One. 2014;9(10):e108896.
30. Torroni A, Huoponen K, Francalacci P, Petrozzi M, Morelli L, Scozzari R, et al. Classification of European mtDNAs from an analysis of three European populations. Genetics. 1996;144(4):1835-50.
31. Soto-Hermida A, Fernández-Moreno M, Oreiro N, Fernández-López C, Pértega S, Cortés-Pereira E, et al. Mitochondrial DNA (mtDNA) haplogroups influence the progression of knee osteoarthritis. Data from the Osteoarthritis Initiative (OAI). PloS one. 2014;9(11):e112735.
32. Fang H, Zhang F, Li F, Shi H, Ma L, Du M, et al. Mitochondrial DNA haplogroups modify the risk of osteoarthritis by altering mitochondrial function and intracellular mitochondrial signals. Biochimica et biophysica acta. 2016;1862(4):829-36.
33. Hildyard JC, Ammälä C, Dukes ID, Thomson SA, Halestrap AP. Identification and characterisation of a new class of highly specific and potent inhibitors of the mitochondrial pyruvate carrier. Biochim Biophys Acta. 2005;1707(2-3):221-30.
34. Rupp H, Zarain-Herzberg A, Maisch B. The use of partial fatty acid oxidation inhibitors for metabolic therapy of angina pectoris and heart failure. Herz. 2002;27(7):621-36.
35. Pascual Garrido C, Hakimiyan AA, Rappoport L, Oegema TR, Wimmer MA, Chubinskaya S. Anti-apoptotic treatments prevent cartilage degradation after acute trauma to human ankle cartilage. Osteoarthritis and cartilage. 2009;17(9):1244-51.
36. Lane RS, Fu Y, Matsuzaki S, Kinter M, Humphries KM, Griffin TM. Mitochondrial respiration and redox coupling in articular chondrocytes. Arthritis Res Ther. 2015;17(1):54.
37. Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. Cell Metab. 2017;25(5):1027-36.
38. Smith RL, Soeters MR, Wüst RCI, Houtkooper RH. Metabolic Flexibility as an Adaptation to Energy Resources and Requirements in Health and Disease. Endocr Rev. 2018;39(4):489-517.
39. Abdelrazig S, Ortori CA, Doherty M, Valdes AM, Chapman V, Barrett DA. Metabolic signatures of osteoarthritis in urine using liquid chromatography-high resolution tandem mass spectrometry. Metabolomics. 2021;17(3):29.
40. Zou S, Zhu L, Huang K, Luo H, Xu W, He X. Adipose tissues of *MPC1*(±) mice display altered lipid metabolism-related enzyme expression levels. PeerJ. 2018;6:e5799.
41. Herzig S, Raemy E, Montessuit S, Veuthey JL, Zamboni N, Westermann B, et al. Identification and functional expression of the mitochondrial pyruvate carrier. Science. 2012;337(6090):93-6.
42. Dalmao-Fernández A, Hermida-Gómez T, Lund J, Vazquez-Mosquera ME, Rego-Pérez I, Garesse R, et al. Mitochondrial DNA from osteoarthritic patients drives functional impairment of mitochondrial activity: a study on transmitochondrial cybrids. Cytotherapy. 2021;23(5):399-410.

8. APPENDIX-Ⅰ：

Protocol for Use with LightCycler® 480 Multiwell Plate 96

The following table shows the PCR parameters that must be programmed for a LightCycler® 480 System PCR run with the LightCycler® 480 SYBR Green I Master using a LightCycler® 480 Multiwell Plate 96:

Setup					
Block Type		Reaction Volume [µl]			
96		10 – 100			
Detection Format		Excitation Filter		Emission Filter	
SYBR Green / HRM Dye		465		510	
Programs					
Program Name		Cycles		Analysis Mode	
Pre-Incubation		1		None	
Amplification		45 [1]		Quantification	
Melting Curve		1		Melting Curve	
Cooling		1		None	
Temperature Targets					
	Target [°C]	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisitions [per °C]
Pre-Incubation	95	None	00:05:00 [2]	4.4	–
Amplification	95	None	00:00:10	4.4	–
	primer dependent [3]	none	00:00:05 - 00:00:20 [4]	2.2 (Target °C ≥ 50°C) 1.5 (Target °C < 50°C) [7]	–
	72	single	00:00:05 - 00:00:30 [4] [5]	4.4	–
Melting Curve [8]	95	None	00:00:05	4.4	–
	65	None	00:01:00	2.2	–
	97	Continuous	–	–	5 – 10 [6]
Cooling	40	None	00:00:10	1.5	–

9. APPENDIX-Ⅱ：

The average score used in the study of the samples to categorize the grade of cartilage deterioration, extracted from Pascual Garrido *et al.,* 2009 (35).

I Structure	Grade
Normal	0
Superficial layer disrupted	1
Superficial layer absent	2
Irregular surface	3
Fissures into the middle zone	4
Slight disorganization	5
Fissures into the deep zone	6
Chaotic disorganization	7
II Cellular abnormalities	
Normal	0
Hypocellularity	1
Large Clones	2
III Matrix staining	
Normal	0
Staining reduced in middle layer	1
Staining reduced in territorial matrix of middle or deep layer	2
Staining present in the interterrritorial matrix and reduced in the territorial matrix	3
Staining reduced in both territorial and interterritorial matrices	4
Maximum score	13