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Effects of the oral administration of glycosaminoglycans with or without native type II collagen on the articular cartilage transcriptome in an osteoarthritic-induced rabbit model

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Abstract

Background In a previous study, the 84-day administration of glycosaminoglycans (GAGs), with or without native collagen type II (NC), in an osteoarthritis (OA)-induced rabbit model slowed down OA progression, improved several micro- and macroscopic parameters and magnetic resonance imaging (MRI) biomarkers in cartilage, and increased hyaluronic acid levels in synovial fluid. To elucidate the potential underlying mechanisms, a transcriptomics approach was conducted using medial femoral condyle and trochlea samples.

Results The administration of chondroitin sulfate (CS), glucosamine hydrochloride (GlHCl), and hyaluronic acid (HA), with (CGH-NC) or without (CGH) NC, strongly modulated several genes involved in chondrocyte extracellular matrix (ECM) remodeling and homeostasis when compared to non-treated rabbits (CTR group). Notably, both treatments shared the main mechanism of action, which was related to ECM modulation through the down-regulation of genes encoding proteolytic enzymes, such as ADAM metallopeptidase with thrombospondin type 1 motif, 9 (*Adamts9*), and the overexpression of genes with a relevant role in the synthesis of ECM components, such as aggrecan (*Acan*) in both CGH-NC and CGH groups, and fibronectin 1 (*Fn1)* and collagen type II, alpha 1 (*Col2A1)* in the CGH group. Furthermore, there was a significant modulation at the gene expression level of the mTOR signaling pathway, which is associated with the regulation of the synthesis of ECM proteolytic enzymes, only in CGH-NC-supplemented rabbits. This modulation could account for the better outcomes concerning the microscopic and macroscopic evaluations reported in these animals.

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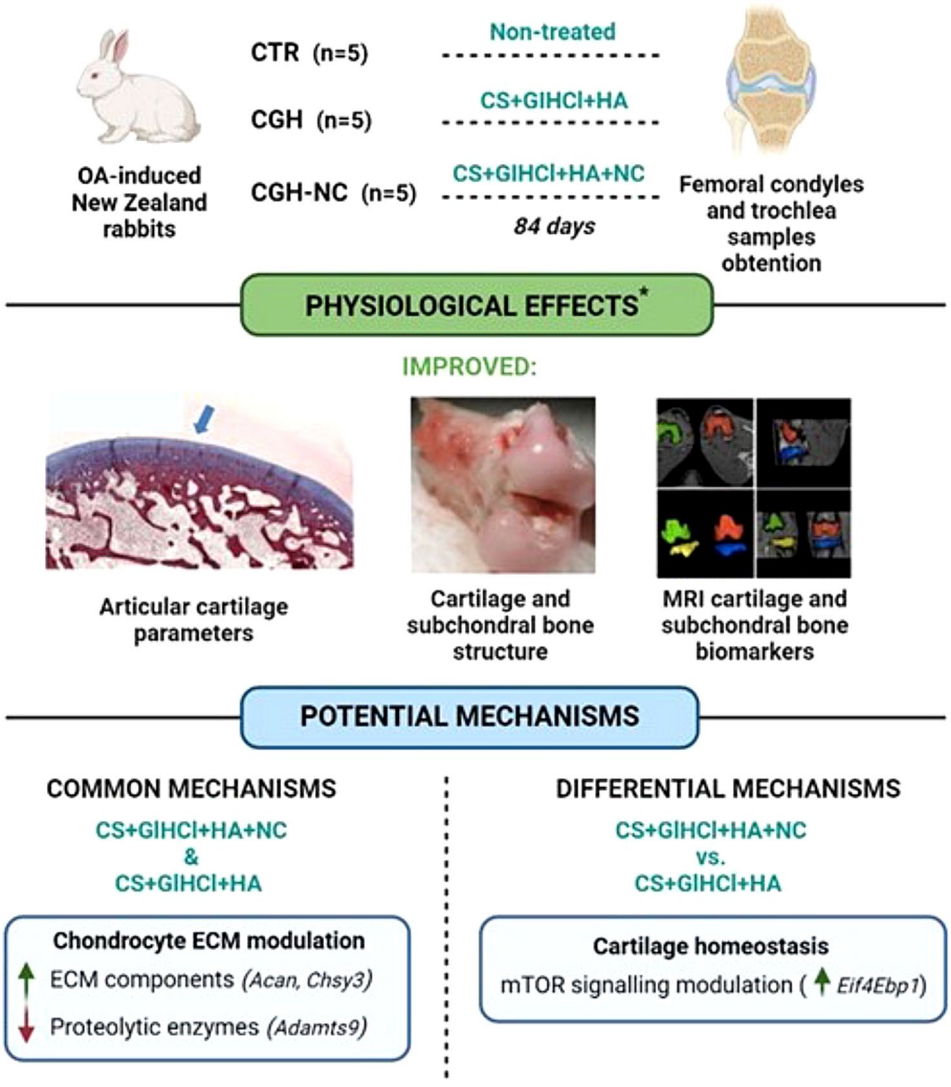
Conclusions In conclusion, the expression of key genes involved in chondrocyte ECM remodeling and homeostasis was significantly modulated in rabbits in response to both CGH and CGH-NC treatments, which would partly explain the mechanisms by which these therapies exert beneficial effects against OA.

Graphical Abstract



Keywords Transcriptomics, Osteoarthritis, Articular cartilage, Native type II collagen, Nutraceuticals

Introduction

Osteoarthritis (OA) is one of the most prevalent joint diseases, significantly contributing to frailty, disability, and socioeconomic burden [1]. As established by the Osteoarthritis Research Society International, OA manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodelling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness [2, 3]. Although several efforts have been made in the study of various pharmaceutical and nutraceutical treatments aimed at slowing the structural deterioration seen in OA cartilage, there is still a lack of well-established approaches to effectively improve the clinical signs of this disease [1, 4]. Additionally, there are no disease-modifying drugs approved by regulatory agencies, and the therapeutic approaches are mainly focused on symptomatic treatment. Thus, gaining a deeper understanding of the mechanisms underlying the onset and progression of OA remains crucial.

Among the different drugs or supplements administered to manage OA symptoms, chondroitin sulfate (CS),

glucosamine hydrochloride (GlHCl) and hyaluronic acid (HA) are recognized as symptomatic slow-acting drugs for OA, having shown beneficial effects in OA patients, such as pain relief and improvement of joint function [5, 6]. In addition, native type II collagen (NC) has also been reported as an effective tool for the management of OA, thanks to its specific immune-mediated mechanism of action, which inhibits inflammation and tissue catabolism at the articular level [7, 8]. Interestingly, NC has also been shown to exert beneficial effects as a pain reliever in a rat model of osteoarthritis [9].

In a previous study carried out by members of our team [10], the administration of a combination of GAGs (CS, GlHCl and HA) with or without NC was evaluated in an OA-induced model by cranial cruciate ligament transection in rabbits. Notably, the oral administration of both treatments for 84 days significantly improved several macroscopic and microscopic parameters related with the degenerative state of the articular cartilage, such as cartilage structure integrity and chondrocyte density [10]. Similar results were observed in the microscopic evaluation of subchondral bone and synovial membrane. Additionally, adding NC to a combination of GAGs showed a greater improvement in magnetic resonance imaging (MRI) biomarkers in the articular cartilage and subchondral bone, reduced inflammatory changes in the synovial membrane, and a higher content of hyaluronic acid in the synovial fluid, compared to GAGs supplementation alone [10] (Supplementary Table 1).

Transcriptomics approaches, including gene microarray analysis, have emerged as highly effective approaches for investigating the underlying mechanisms of complex diseases such as OA, being valuable in identifying specific genes and pathways implicated in the progression of this disease [11]. These studies not only enhance our understanding of the mechanisms contributing to OA but also can open new directions for potential pharmaceutical and nutraceutical therapies tailored to address this condition [11, 12].

Therefore, the main aim of this study was to gain insights into the genes and potential pathways involved in the beneficial effects observed after the 84-day supplementation of GAGs, with or without NC, in OA-induced rabbits by performing a microarray analysis on medial femoral condyle and trochlea samples.

Materials and methods

Experimental design

The same cohort of fifteen, twelve-week-old female New Zealand rabbits used in Sifre et al. [10] were included in the present study. Cranial cruciate ligament transection (CCLT) was performed to induce OA on the right stifle. The day after CCLT surgery, the rabbits were divided into three groups ($n=5$), based on the treatment received for 84 days: a control group with no treatment (CTR), a group treated with the combination of CS (CS Bioactive®), GlHCl and HA (Mobilee®) (CGH) and a group treated with CS, GlHCl and HA with NC (Collavant n2®) (CGH-NC). All products were manufactured and provided by Bioiberica, S.A.U. (Esplugues de Llobregat, Spain). The administered doses of each compound were the following: CGH (60.38 mg/kg CS+75.47 mg/kg GlHCl+3.35 mg/kg HA) and CGH-NC (60.38 mg/kg CS+75.47 mg/kg GlHCl+3.35 mg/kg HA+0.67 mg/kg NC). All treatments were administered orally in a daily basis by the same researcher, diluting them in a 2-mL syringe with tap water. Doses were weekly adjusted depending on the animal's weight. On day 84, animals were euthanized, and the femoral condyles were carefully dissected and cut longitudinally using a manual saw separating the lateral condyle from the trochlear section and the medial femoral condyle. The lateral condyle was used for different investigation purposes not related to the current study. Once the trochlear and medial condyle sections were cut, the cartilage of both the trochlear and medial condyle was meticulously collected and preserved at -80ºC until further analysis. These tissues were collected in proximity to the lesion site produced in the CCLT intervention. All procedures were performed according to the European legislation on protection of animals and with the approval of the Local Government Animal Protection Ethics Committee (RD53/2013).

Total RNA isolation

RNA extraction was performed with RNeasy Lipid Tissue Mini Kit (Qiagen, P/N 74804) according to instructions from manufacturer, and amount and quality of the RNA checked by Bioanalyzer 2100 (Agilent Technologies). For each sample, ss-cDNA was synthesized from 2 ng of total RNA using the GeneChip WT Pico Reagent kit (Affymetrix ThermoFisher Scientific, P/N 703262), according to the protocol supplied by manufacturer. The amount and quality of ss-cDNA was checked by Nanodrop 2000 (ThermoFisher Scientific) and Bioanalyzer. ss-cDNA targets were cleaned up and after fragmentation and terminal labelling, 5.5 µg of fragmented and biotinylated ss-DNA were included in the hybridization mix, using the GeneChip Hybridization, Wash and Stain (Affymetrix ThermoFisher Scientific, P/N 900720) according to recommendations of manufacturer and the resulting preparations were hybridized to GeneChip™ Rabbit Gene 1.0 ST Array (Affymetrix, ThermoFisher Scientific, P/N 902238) with almost 500,000 probes that provide a comprehensive coverage of the whole transcriptome (23,280 genes).

Microarray processing

After scanning using a GeneChip3000 with autoloader, microarrays data were processed using Affymetrix

Expression Command Console (Affymetrix). Data analyses were then performed with RMA (Robust Multiarray Average) allowing that raw intensity values were background corrected, log2 transformed and then quantile normalized in order to obtain an individual intensity value for each probe set. All samples passed hybridization and labelling quality controls. TAC software (Transcriptome Analysis Console) from ThermoFisher Scientific and Partek Genomics Suite and Partek Pathways softwares (Partek Incorporated, St. Louis, USA) were used for the statistical analysis.

Microarray data analysis

For the transcriptomics analyses, correction for multiple testing were not employed, as these corrections are frequently too strict to detect the subtle effects typically encountered in nutritional studies. However, we defined the threshold of significance at $p=0.005$ instead of $p=0.05$ and, therefore, only those genes with a p-value <0.005 were considered to avoid interferences of secondary, false altered metabolic pathways.

Volcano plots of the microarray results were made by using GraphPad Prism version 10 (Graphpad Software, San Diego, CA, USA). Gene symbols and fold-changes were entered in Joint-Pathway Analysis module from Metaboanalyst 5.0 [11], selecting the Fisher's exact test as enrichment algorithm and a degree centrality measurement (number of links that connect to a node) as topology analysis. Significant pathways with $p<0.05$ were selected to assess the mechanisms affected during OA and modulated by the different treatments. In addition, a heatmap analysis was performed with the top 50 Differentially expressed genes (DEGs) of the different comparisons carried out in the present study, normalizing data using the Pareto Scaling in Metaboanalyst. To explore the role of treatments in deep, the full list of genes involved in OA progression and severity reported in the literature was obtained from DisGeNET 7.0 [12]. This full gene list was compared with the full affected genes in each comparison with treatments in rabbit models. Those significantly changed genes ($p<0.005$) that matched with the OA gene list were further discussed.

Table 1 Nucleotide sequences of primers used for PCR amplification in rabbit samples

Gene	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)
Ppia	TCCAGGGTTTATGTGCCAGGGTG	CGTTTGCCATGGACAAGATGCC
Acan	CGCTACTCACTGACCTTCGA	GGGCTCACAATGGGGTATCT
Wwp2	GACGCACAGACATTCAGGTG	AGGTTCTTCGGGATCAGTGG
Eif4ebp1	CTCCCTTCCTCTCTGTCACC	GAAGAGCCAGACATCCCTCA

Real-time-quantitative PCR gene expression analysis

The mRNA expression levels of aggrecan (*Acan*), WW domain containing E3 ubiquitin protein ligase 2 (*Wwp2*) and Eukaryotic translation initiation factor 4E binding protein (*Eif4ebp1*) were assessed by real-time quantitative RT-polymerase chain reaction (RT-qPCR) analysis in the rabbit samples used for the microarray study to validate the data analysis.

The total RNA of the medial femoral condyle and trochlea samples was supplied by Bioiberica, S.A.U. The RNA yield was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). To analyse the expression of the samples, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Barcelona, Spain). A MyGene L Series Peltier Thermal Cycler (LongGene, Hangzhou, China) was used for reverse transcription. The reaction was performed according to the instructions of the manufacturer. The cDNA was subjected to a quantitative reverse transcriptase polymerase chain reaction amplification using LightCycler 480 SYBR green I Master-20 (Roche Diagnostics, Barcelona, Spain) in a Light Cycler 480II (Roche Diagnostics, Barcelona, Spain). The primers used for the different genes were obtained from Biomers.net (Ulm, Germany) and are described in the Table 1. All genes (both the constitutive and the tested genes) were analysed in triplicate for each sample. The fold changes in the mRNA levels were calculated using the $-2^{\Delta\Delta Ct}$ method [13] with peptidylprolyl isomerase A (*Ppia*) gene as endogenous control.

Statistical analysis

In the real-time-quantitative PCR gene expression results, data are expressed as the mean ± standard error of the mean (SEM). Grubbs' test was used to detect outliers. Student's t-test was used for single statistical comparisons. Statistical analyses were performed with SPSS Statistics 26.0 (SPSS, Inc., Chicago, IL, USA), and the level of statistical significance was set at bilateral 5% ($p<0.05$).

The statistical approach of the microarray data has been previously described in the microarray data analysis section.

Results

DEGs between CGH and CTR groups

The microarray analysis performed on the medial femoral condyle and trochlea samples extracted on day 84 in CGH and CTR rabbits reported a total of 466 genes that were significantly differentially expressed between both groups ($p<0.005$, Student's t test), as shown in the Volcano plot (Fig. 1A). Specifically, the expression of 220 and 246 genes were significantly up- and

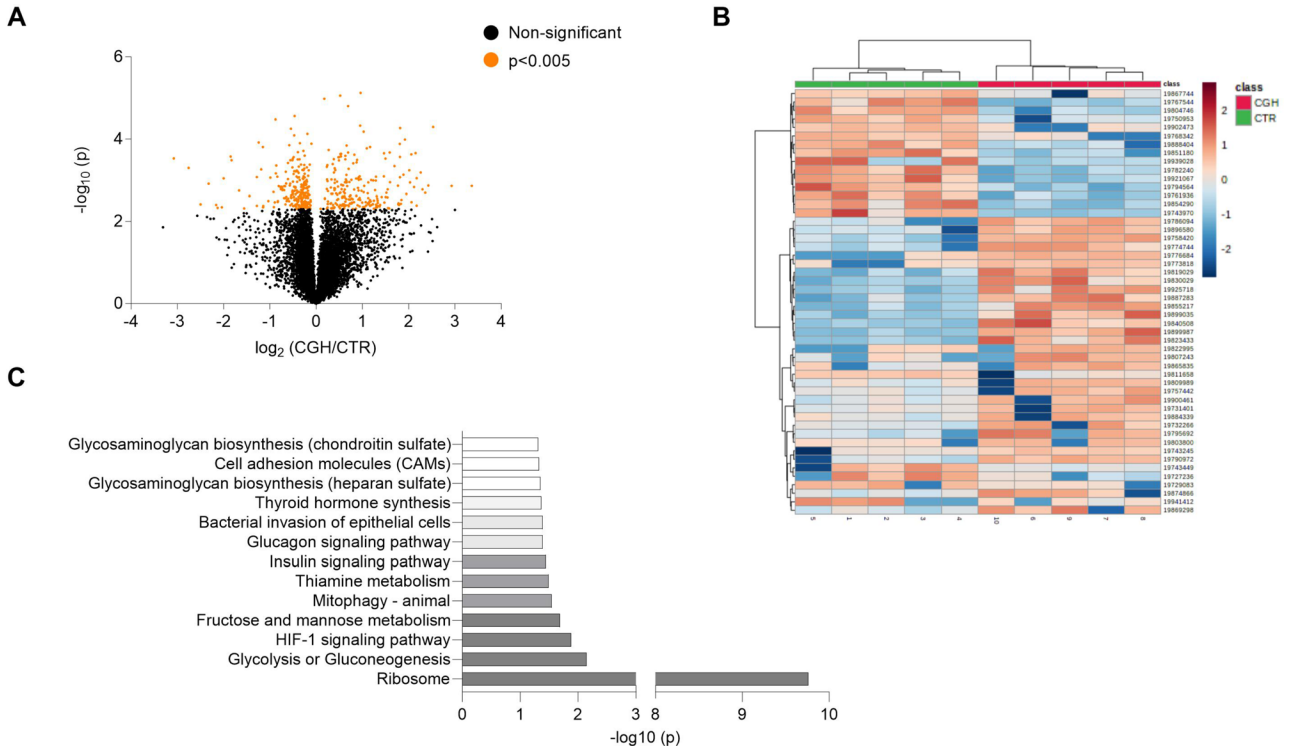


Fig. 1 **(A)** A Volcano plot analysis was performed in order to represent the 466 differentially expressed genes (DEGs) between CGH and CTR rabbit groups. Genes that were statistically significant ($p < 0.005$) are depicted as orange dots. **(B)** Based on the top 50 DEGs between CGH and CTR groups, a heatmap analysis was performed, in which all 10 animals (5 CGH and 5 CTR) were hierarchical clustered between both groups. Data were normalized using the Pareto Scaling in Metaboanalyst. **(C)** A joint pathway analysis was performed with those up- and down-regulated DEGs with a p-value < 0.005, obtaining 13 statistically significant metabolic pathways

down-regulated, respectively, in CGH compared to CTR rabbits. A heatmap analysis based on the top 50 DEGs (Student's *t* test) showed clear hierarchical clustering between the two groups (Fig. 1B). The 466 genes were included in a joint pathway analysis to identify the most relevant pathways potentially modulated by CGH treatment. A total of 13 metabolic pathways, including the HIF-1 signalling pathway, glycosaminoglycan biosynthesis and glucose-related metabolism pathways, among others, were found to be significantly affected (Fig. 1C).

Regarding OA-related genes, a list of the most affected genes ($p < 0.005$) involved in OA pathogenesis in CGH rabbits compared to CTR group has been represented in Table 2. Interestingly, some genes encoding proteins that are components of the articular cartilage extracellular matrix (ECM) were upregulated in CGH-treated rabbits, such as *Col2A1*, *Acan* and *Fn1* (Table 2).

DEGs between CGH-NC and CTR groups

Microarray data obtained from medial femoral condyle and trochlea samples showed a total of 230 genes differently expressed between CGH-NC and CTR groups ($p < 0.005$, Student's *t* test), as depicted in the Volcano plot (Fig. 2A). Among these differentially expressed genes, 105 and 125 genes were significantly up- and down-regulated, respectively, in CGH-NC compared to CTR rabbits. A heatmap analysis based on the top 50 DEGs (Student's *t* test) demonstrated clear hierarchical clustering between both groups (Fig. 2B). The 230 genes underwent a joint pathway analysis to identify the most relevant pathways potentially modulated by CGH-NC treatment. A total of 10 metabolic pathways, including the adipocytokine signalling pathway, cell adhesion molecules and lipid metabolism-related pathways, showed significant changes (Fig. 2C).

Regarding OA-related genes, a list of the most affected genes ($p < 0.005$) involved in OA pathogenesis in CGH-NC rabbits compared to CTR group has been represented in Table 3. Genes encoding proteins that are components of the articular cartilage ECM, such as *Acan*, and genes involved in cartilage homeostasis, such as *Wwp2*, were significantly up-regulated in CGH-NC-treated rabbits, in comparison with CTR rabbits (Table 3).

DEGs between CGH-NC and CGH groups

Regarding the gene expression analysis performed in medial femoral condyle and trochlea samples extracted at day 84 in CGH-NC- and CGH-treated rabbits, a total

Table 2 DEGs involved in OA pathogenesis comparing CGH vs. CTR groups

Gene Symbol	Associated Protein	*p*-value	Fold-Change	UP/ DOWN
Fgf10	Fibroblast growth factor 10	0.004	-1.300	DOWN
Adamts9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	0.002	-1.163	DOWN
Med12l	Mediator complex subunit 12-like	0.004	-1.158	DOWN
Chsy3	Chondroitin sulfate synthase 3	0.002	1.195	UP
Znf410	Zinc finger protein 410	0.005	1.315	UP
Socs2	Suppressor of cytokine signalling 2	0.003	1.662	UP
Il16	Interleukin 16	0.0002	1.772	UP
Col2A1	Collagen, type II, alpha 1	0.004	2.196	UP
Aldh6A11	Aldehyde dehydrogenase 6 family, member A1	0.002	2.479	UP
Acan	Aggrecan	0.003	2.495	UP
Fn1	Fibronectin 1	0.004	2.513	UP
S100A1	S100 calcium binding protein A1	0.002	2.864	UP
S100A6	S100 calcium binding protein A6	0.0001	3.518	UP

OA pathogenesis-related DEGs ($p<0.005$) in CGH rabbits compared to CTR rabbits (Student's t test), sorted by Fold-Change

of 87 genes were significantly differentially expressed between both groups ($p<0.005$, Student's t test), as shown in the Volcano plot (Fig. 3A). Specifically, the expression of 55 and 32 genes were significantly up- and down-regulated, respectively, in CGH-NC compared to CGH rabbits. A heatmap analysis based on the top 50 DEGs (Student's t test) showed clear hierarchical clustering between both groups (Fig. 3B). The 87 genes were included in a joint pathway analysis to identify which were the most relevant pathways that could be modulated by CGH-NC treatment compared to CGH. A total of 7 metabolic pathways, including ECM-receptor interaction, mTOR and PPAR signalling pathways, among others, showed significant changes (Fig. 3C).

Regarding OA-related genes, a list of the most affected genes ($p<0.005$) involved in OA pathogenesis in CGH-NC rabbits compared to CGH rabbits has been represented in Table 4.

Common DEGs between CGH-NC and CGH groups vs. CTR group

Finally, the common DEGs in CGH-NC and CGH groups between CTR animals were analysed. As shown in Fig. 4A, a total of 58 common DEGs ($p<0.005$) were identified in both CGH-NC and CGH groups versus CTR rabbits and represented in a Venn diagram. The expression of 27 and 31 genes were significantly up- and down-regulated, respectively, in both CGH-NC and CGH compared to CTR. Importantly, the expression changes in all genes were consistent in both groups (Fig. 4B). The 58 genes were included in a joint pathway analysis to identify which were the most relevant pathways that could be commonly modulated by both CGH-NC and CGH treatments. A total of 3 pathways, including glycosaminoglycan biosynthesis, among others, showed significant p-values (Fig. 4C).

Regarding OA-related genes, a list of the most affected common genes ($p<0.005$) involved in OA pathogenesis in both CGH-NC and CGH groups has been represented in Table 5. As previously reported, genes encoding proteins that are components of the articular cartilage ECM, such as *Acan*, and genes encoding proteolytic enzymes, such as *Adamts9*, were significantly up- and down-regulated, respectively, in both CGH-NC-and CGH-treated rabbits, in comparison with CTR rabbits.

Validation of array results by RT-qPCR

RT-qPCR analysis was performed in order to validate the results obtained in the array analysis. A total of three genes (*Acan*, *Wwp2 and Eif4ebp1*) that were differentially expressed among the three experimental groups were selected for this analysis. As shown in Table 6, the results of the RT-qPCR analysis served to corroborate the findings from the array data, revealing significant differences in the gene expression patterns in two out of the three analysed genes (*Acan* and *Wwp2)*. Regarding *Eif4ebp1*, the variations detected in the array analysis between the CGH-NC and CGH groups were not corroborated by RT-qPCR (Student t-test, $p=0.436$), although a comparable fold change was evident in both methodologies (Table 6).

Discussion

OA is a complex and multifactorial disease characterized by the degeneration of joint cartilage and the underlying bone. The degradation of cartilage in this disease involves several molecular processes, including the action of MMPs and aggrecanase, inflammation, oxidative stress, chondrocyte apoptosis and autophagy dysregulation, among others [14]. Therefore, the main supplements or

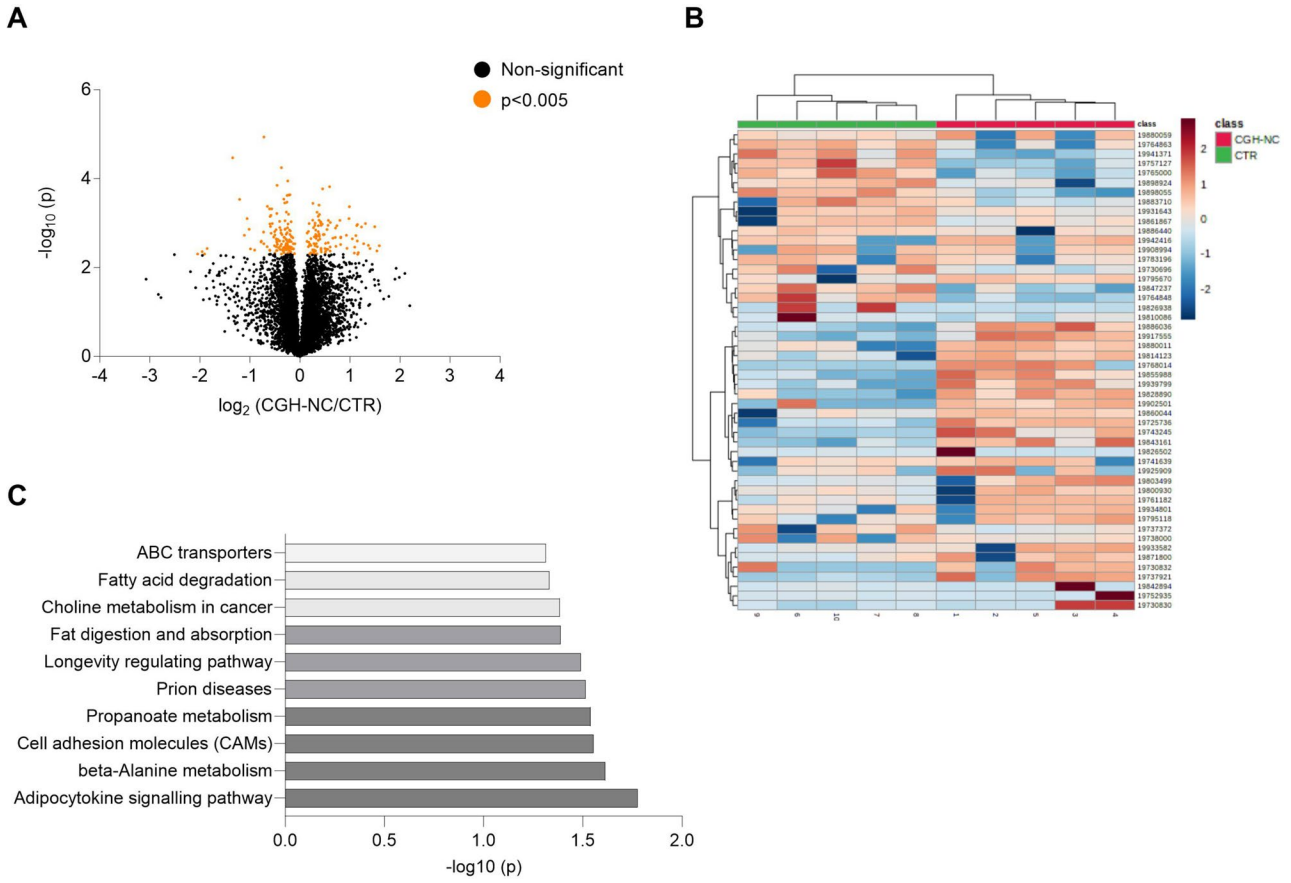


Fig. 2 **(A)** A Volcano plot analysis was performed in order to represent the 230 differentially expressed genes (DEGs) between CGH-NC and CTR rabbit groups. Genes that were statistically significant ($p < 0.005$) are depicted as orange dots. **(B)** Based on the top 50 DEGs between CGH-NC and CTR groups, a heatmap analysis was performed, in which all 10 animals (5 CGH-NC and 5 CTR) were hierarchical clustered between both groups. Data were normalized using the Pareto Scaling in Metaboanalyst. **(C)** A joint pathway analysis was performed with those up- and down-regulated DEGs with a p-value < 0.005, obtaining 10 statistically significant metabolic pathways

nutraceuticals on the market aimed at the prevention and treatment of OA are focused on improving these processes. As an example, glucosamine sulfate is a normal component of glycosaminoglycans in the cartilage matrix and synovial fluid, which has been described to act through the maintenance of mechanical properties of the cartilage, the inhibition of proteolytic enzymes and the promotion of GAGs and proteoglycan synthesis. Additional mechanisms of action include anti-inflammatory, immunosuppressive and anticatabolic properties [15]. In OA, native type II collagen has been described to act through an oral tolerance mechanism of action, in which low doses can exert an immunomodulatory effect that supresses the T-cell-mediated destruction of type II collagen in joint tissue [16]. Additionally, hyaluronic acid and/or native type II collagen supplementation beneficial effects on OA have been related with a wide range of mechanisms of action, including chondroprotective effects, proteoglycan or GAG synthesis and anti-inflammatory effects, among others [17, 18].

In the present study, the transcriptomic approach revealed several potential mechanisms involved in the previously reported beneficial effects against OA from the administration of CS with GlHCl and HA, with or without NC, in OA-induced rabbits [10]. To analyse in depth the modulatory capacity of the different treatments evaluated, we carried out various comparative approaches among the experimental groups.

In the joint pathway analysis carried out with the DEGs reported in CGH rabbits compared to their non-treated counterparts, the HIF-1 signalling pathway emerged as one of the most significantly affected metabolic pathways. Chondrocytes exist in a low-oxygen environment, where the hypoxia-inducible factor 1 alpha (*Hif-1α*) signalling pathway plays a pivotal role in responding to decreased oxygen levels [19, 20]. Interestingly, the activation of this protein in response to reduced oxygen levels in this tissue is associated with the regulation of autophagy and apoptosis, inhibition of inflammatory cytokine synthesis, and the maintenance of the chondrocyte ECM environment. Furthermore, HIF-1α can maintain the

Table 3 DEGs involved in OA pathogenesis comparing CGH-NC vs. CTR groups

Gene Symbol	Associated Protein	*p*-value	Fold-Change	UP/ DOWN
AdipoR2	Adiponectin receptor 2	0.003	-1.372	DOWN
Fgf10	Fibroblast growth factor 10	0.0001	-1.367	DOWN
Chi3l2	Chitinase 3-like 2	0.005	-1.152	DOWN
Adamts9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	0.005	-1.127	DOWN
Chsy3	Chondroitin sulfate synthase 3	0.002	1.195	UP
Agap3	ArfGAP with GTPase domain, ankyrin repeat and PH domain 3	0.004	1.240	UP
FoxS1	Forkhead box S1	0.002	1.316	UP
Aldh6A1	Aldehyde dehydrogenase 6 family, member A1	0.001	1.450	UP
Nfat5	Nuclear factor of activated T-cells 5, tonicity-responsive	0.002	1.588	UP
Il16	Interleukin 16	0.003	1.751	UP
Acan	Aggrecan	0.003	1.951	UP
Wwp2	WW domain containing E3 ubiquitin protein ligase 2	0.004	2.144	UP

OA pathogenesis-related DEGs ($p<0.005$) in CGH-NC rabbits compared to CTR rabbits (Student's t test), sorted by Fold-Change

chondrogenic phenotype, which regulates glycolysis — the primary source of energy for chondrocytes and one of the most significantly modulated pathways in CGH rabbits [19, 20].

In addition, the CGH group displayed a significant up-regulation of *Col2A1*, which encodes collagen type II, the predominant component of the cartilage matrix. Although this protein was primarily viewed as a component that provides structural support to the cartilage matrix, recent findings have highlighted its role as an extracellular signalling molecule with the capacity to prevent chondrocyte hypertrophy, one of the main processes associated with OA progression [21].

Fibronectin is a ubiquitous glycoprotein found in the ECM, primarily serving as a substrate for cell attachment and playing a crucial role in communication between the intra and the extracellular environment [22]. Fibronectin is abundant in normal articular cartilage, produced by chondrocytes to assemble a functional cartilage matrix [23]. In OA, proteolytic fragments of fibronectin are increased in the altered matrix and stimulate its catabolism, being involved in the promotion of cartilage degeneration [24]. Nevertheless, intact fibronectin has been described to exert different effects on ECM homeostasis, contributing to the maintenance or repair of articular cartilage ECM via stimulation of proteoglycan synthesis [22]. In the present study, CGH-supplemented animals displayed a significant increase in the gene expression levels of *Fn1*. This result could be interpreted as a mechanism aimed at counteracting the degradation of the articular cartilage ECM reported in OA-induced rabbit model, through the synthesis of ECM components. Considering that gene expression levels do not always match protein data, additional measurements focused on the analysis of the protein levels of these key ECM components would be useful to validate these results.

Collagen type II is the main structural component of the cartilage tissue, playing a significant role in the mechanical integrity of the cartilage, regulating chondrocyte differentiation, and maintaining cartilage physiological homeostasis [25]. In this study, the supplementation of CS, GlHCl, and HA, in combination with Collavant n2®, a trusted source of quality native (undenatured) type II collagen extracted from chicken sternum, was evaluated.

The most significant metabolic pathway identified in the joint pathway analysis performed with the DEGs in CGH-NC compared to the CTR group was the adipocytokine signalling pathway. Adipocyte-derived molecules, known as adipokines, have gained interest in OA pathophysiological research due to their role in cartilage and bone homeostasis [26]. For example, adiponectin has been described to be implicated in OA pathogenesis, with higher plasma and synovial fluid levels found in OA patients compared to healthy individuals [27]. In chondrocytes, adiponectin acts through AdipoR1 and AdipoR2, increasing MMP-1, MMP-3 and MMP-13 [27]. Interestingly, in the present study, CGH-NC rabbits exhibited a significant down-regulation of adiponectin receptor 2 (*AdipoR2*). Other pathways related to lipid metabolism, such as fat digestion and absorption, and fatty acid degradation, were also significantly modulated in the CGH-NC group, although they do not seem to be directly connected to the reported beneficial effects on cartilage structure in this group.

Interestingly, CGH-NC rabbits displayed a significant down-regulation of chitinase 3-like 2 (*Chi3l2*), a gene that has been shown to be up-regulated in osteoarthritic cartilage and contribute to OA progression by activating an autoimmune response and participating in chondrocyte remodelling [28]. Thus, it is considered a robust marker of chondrocyte differentiation and OA pathogenesis [29].

Aggrecanase-mediated aggrecan degradation in synovial fluid is a significant event that occurs in the early stages of OA, making it a relevant target for OA treatments aimed at slowing the destructive process by enhancing aggrecan production and inhibiting its

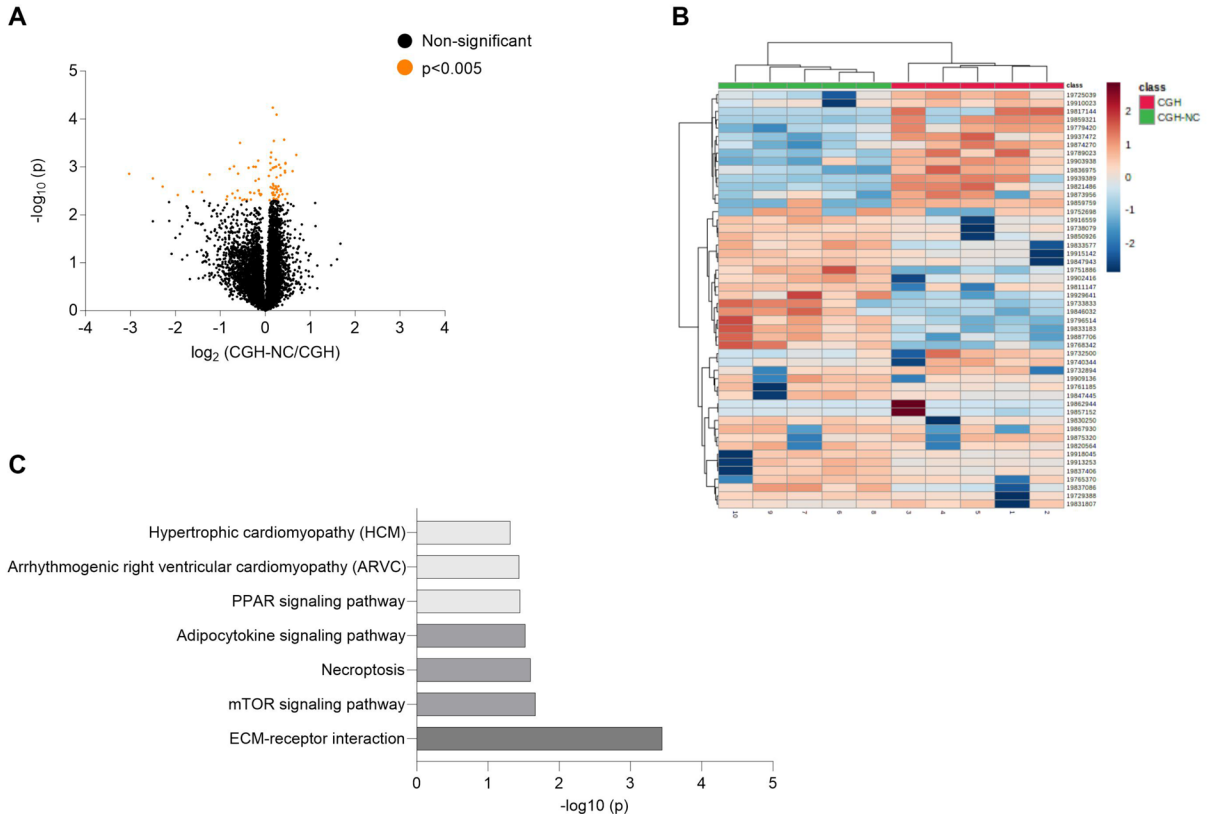


Fig. 3 **(A)** A Volcano plot analysis was performed in order to represent the 87 differentially expressed genes (DEGs) between CGH-NC and CGH rabbit groups. Genes that were statistically significant ($p < 0.005$) are depicted as orange dots. **(B)** Based on the top 50 DEGs between CGH-NC and CGH groups, a heatmap analysis was performed, in which all 10 animals (5 CGH-NC and 5 CGH) were hierarchical clustered between both groups. Data were normalized using the Pareto Scaling in Metaboanalyst. **(C)** A joint pathway analysis was performed with those up- and down-regulated DEGs with a p-value < 0.005, obtaining 7 statistically significant metabolic pathways

Table 4 DEGs involved in OA pathogenesis comparing CGH-NC vs. CGH groups

Gene Symbol	Associated Protein	*p*-value	Fold-Change	UP/ DOWN
Cd36	CD36 molecule (thrombospondin receptor)	0.003	1.082	UP
Syt7	Synaptotagmin VII	0.004	1.123	UP
Itga4	Integrin, alpha 4	0.003	1.150	UP
Itga7	Integrin, alpha 7	0.001	1.153	UP
Trpv5	Transient receptor potential cation channel vanilloid 5	0.003	1.199	UP

OA pathogenesis-related DEGs ($p < 0.005$) in CGH-NC rabbits compared to CGH rabbits (Student's t test), sorted by Fold-Change

degradation [30]. Relevantly, in the present study, we observed that CGH-NC rabbits displayed a significant down-regulation of *Adamts9* gene expression levels and an up-regulation of aggrecan (*Acan*) levels in cartilage samples. This result could directly contribute to the improvement of articular cartilage in CGH-NC rabbits, as described by Sifre et al. [10]. A holistic approach including the transcriptome analysis of synovial membrane would be interesting to corroborate whether these mechanisms are also associated with the beneficial effects of CGH-NC reported in this tissue [10]. Clinical studies have also reported that sodium hyaluronate treatment in patients with knee OA improved clinical symptoms by reducing aggrecan degradation and enhancing its synthesis in the joint tissues [31]. The potential inhibition of aggrecanase-mediated aggrecan degradation reported in CGH-NC rabbits could be induced by the up-regulation of the WW domain containing E3 ubiquitin protein ligase 2 (*Wwp2*) gene, which codes for a protein abundantly expressed in articular cartilage and is proposed to target ADAMTS proteins via poly-ubiquitination and degradation [32]. These results would be aligned with preclinical studies with similar experimental designs, in which the administration of the combination chondroitin sulfate-glucosamine exhibited chondroprotective effects (reduced inflammation and cartilage and bone degradation biomarkers in serum) in an OA model induced by anterior cruciate ligament transection (ACLT) in ovariectomised rats [33].

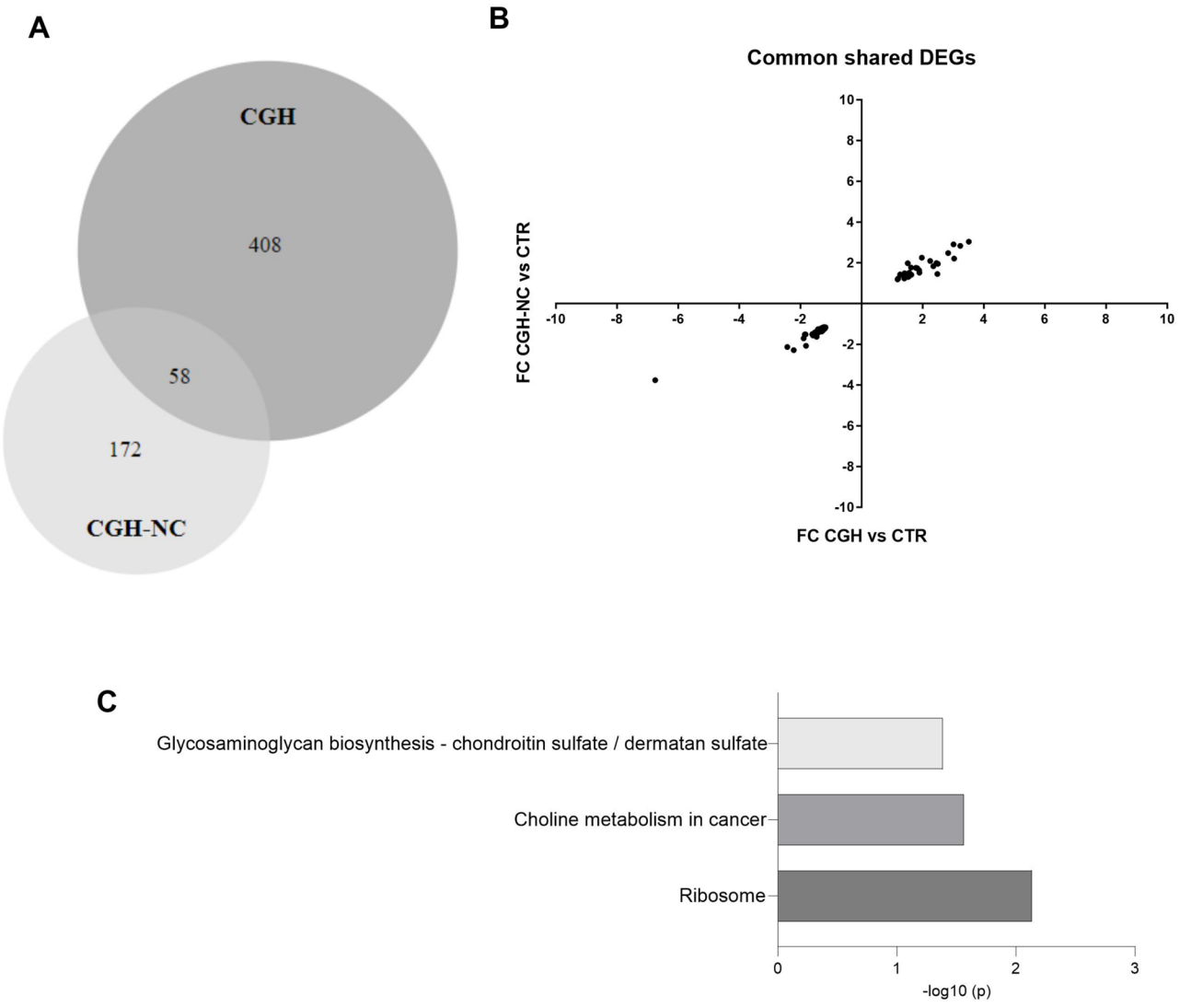


Fig. 4 **(A)** A Venn diagram illustrating the distinct and overlapping sets of significant DEGs ($p < 0.005$) in the context of CGH-NC and CGH groups in comparison to CTR group. **(B)** Representation of the fold changes in those 58 genes commonly shared between CGH-NC and CGH groups. **(C)** A joint pathway analysis was performed with those up- and down-regulated DEGs with a p-value < 0.005, obtaining 3 statistically significant metabolic pathways

Table 5 Common DEGs involved in OA pathogenesis in both CGH and CGH-NC vs. CTR groups

		CGH-NC vs. CTR			CGH vs. CTR		
Gene Symbol	**Associated Protein**	***p*-value**	**Fold-Change**	**UP/ DOWN**	***p*-value**	**Fold-Change**	**UP/ DOWN**
Fgf10	Fibroblast growth factor 10	0.0001	-1.367	DOWN	0.004	-1.300	DOWN
Adamts9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	0.005	-1.127	DOWN	0.002	-1.163	DOWN
Chsy3	Chondroitin sulfate synthase 3	0.004	1.228	UP	0.002	1.195	UP
Il16	Interleukin 16	0.003	1.751	UP	0.0002	1.772	UP
Acan	Aggrecan	0.003	1.951	UP	0.003	2.495	UP
Aldh6a1	Aldehyde dehydrogenase 6 family, member A1	0.001	1.450	UP	0.002	2.479	UP

Common OA pathogenesis-related DEGs ($p < 0.005$) in both CGH-NC and CGH groups (Student's t test), sorted by Fold-Change

All these results pointed out the modulation of aggrecan levels and, consequently, the remodelling and maintenance of ECM homeostasis, as the mechanisms involved in the beneficial effects reported after the administration of CS, GlHCl and HA combination with the addition of NC in the rabbit model of knee OA.

With the main aim of determining whether the addition of NC to the combination of CS, GlHCl and HA

Table 6 Quantitative PCR (qPCR) confirmation of array data for rabbit samples

Gene Symbol	Associated Protein	Groups	Fold-Change array	Fold-Change RT-qPCR	*p*-value array	*p*-value RT-qPCR
Acan	*Aggrecan*	CGH vs. CTR	2.495	10.296	0.003	0.029
		CGH-NC vs. CTR	1.951	2.917	0.003	0.050
Wwp2	*WW domain containing E3 ubiquitin protein ligase 2*	CGH-NC vs. CTR	2.144	6.376	0.004	0.039
Eif4ebp1	*Eukaryotic translation initiation factor 4E binding protein*	CGH-NC vs. CGH	1.340	1.403	0.001	0.436

p-value of the RT-qPCR data is given (Student's t-test, $p < 0.05$)

could induce a differential effect on OA progression, the articular cartilage transcriptome of CGH-NC and CGH were compared. Two major pathways involved in OA pathogenesis were identified: ECM-receptor interaction and the mammalian target of rapamycin (mTOR) signalling pathways.

Specific interactions between the ECM and cells are mediated by transmembrane macromolecular complexes, such as integrins, proteoglycans, CD36 and other cell-surface-associated components, through which regulatory signals and mechanical force are transmitted. These interactions lead to the direct or indirect modulation of several cellular processes, such as migration, differentiation, adhesion and apoptosis [34]. Integrins are a family of transmembrane proteins that interact with ECM molecules, participating in many signalling pathways in chondrocytes, such as cell survival, growth, differentiation, and tissue remodelling [35]. High levels of these proteins have been found in human osteoarthritic cartilage tissues, enhancing ECM deformation and promoting chondrocyte hypertrophy [36]. Unexpectedly, we reported a significant up-regulation of integrin alpha 4 (*Itgα4*) and 7 (*Itgα7*) in CGH-NC rabbits compared to CGH. Similarly, the CD36 gene, which encodes a protein considered a marker of hypertrophic differentiation [37], was also higher in CGH-NC group than in CGH. There are several contradictions regarding the role of these transmembrane proteins in chondrocyte phenotype modulation. Despite evidence of a positive association between integrin levels and OA progression [36, 38], some preclinical studies have reported a protective role of integrin α5 through the inhibition of chondrocyte hypertrophy [39]. Moreover, in vivo studies have shown that the lack of integrin α1 was associated with cartilage degradation and accelerated aging-dependent lesions [40]. Similarly, despite evidence of CD36-mediated promotion of OA via inflammatory processes and chondrocyte hypertrophy [41, 42], Cecil et al. [42] reported that CD36 can induce cartilage repair in response to an inflammatory stimulus, showing chondroprotective properties. Therefore, further studies including protein levels analysis are necessary to understand the relevance of these findings and their association with the macro- and microscopic results observed in both treatments.

Several complex signalling pathways have been proposed to be involved in OA pathogenesis. For example, the mTOR signalling pathway has been described as essential for maintaining joint health, being involved in the three main aspects of OA: cartilage degeneration, synovial inflammation, and subchondral bone sclerosis [43]. Indeed, it has been reported that inhibition of the mTOR signalling pathway mitigates OA joint damage by restoring cartilage homeostasis, suppressing inflammatory responses and enhancing autophagy [43]. In the present study, we observed that CGH-NC rabbits displayed a significant up-regulation of eukaryotic translation initiation factor 4E binding protein (*Eif4Ebp1*), a gene that encodes a member of a family of translational repressor proteins [44]. Relevantly, it has been described that mTOR-mediated inactivation of this gene leads to cartilage degeneration and higher expression of proteolytic enzymes in the articular cartilage of an OA rat model [45], suggesting new potential players involved in cartilage breakdown.

Finally, an analysis of common articular cartilage DEGs between CGH-NC and CGH in comparison with non-treated rabbits was performed. CS is a sulfated GAG found in the ECM that plays a key role in many of the biomechanical properties of cartilage, conferring resistance and elasticity [46]. Lower levels of CS and changes in its structure have been described in osteoarthritic cartilages, being crucial in OA pathogenesis. Therefore, CS has been used as a potential therapeutic intervention to enhance ECM regeneration [46]. Several publications have described CS' beneficial effects on patients with OA by stimulating the synthesis of proteoglycans and hyaluronic acid, inhibiting the synthesis of proteolytic enzymes and other factors associated with ECM degradation, and exerting anti-inflammatory and antioxidant effects in both articular cartilage and synovial membrane [46, 47]. In the present study, both CGH-NC and CGH rabbits exhibited a significant up-regulation of chondroitin sulfate synthase 3 (*Chsy3*) gene expression levels in comparison with CTR rabbits. The fact that both groups received CS as a treatment for 84 days clearly accounted for the higher expression of this gene as part of the enhancement of ECM regeneration.

Moreover, we found some controversies regarding other OA-related genes reported in the present study,

such as interleukin 16 (*Il16*). Both CGH-NC and CGH groups displayed a significant up-regulation of *Il16* gene expression levels. Although a previous in vitro study reported that this interleukin is decreased during chondrogenesis and significantly increased in OA progression [48], another study reported anti-inflammatory properties of this protein, showing its ability to inhibit the production of IL-1β, IFN-γ and TNF-α in the synovium of a mouse model of rheumatoid synovitis [49].

Even though the CS+GlHCl+HA+NC combination administered to CGH-NC rabbits showed better results in the macroscopic evaluation of cartilage appearance and microscopic evaluation of the synovial membrane than the CS+GlHCl+HA treatment administered to CGH group [10], we reported similarities in several OA-related genes. For example, both treated groups exhibited a significant up-regulation of *Acan* and a down-regulation of *Adamts9*, suggesting that both treatments could inhibit aggrecanase-mediated aggrecan degradation, one of the main processes involved in articular cartilage degeneration.

The performance of a microarray analysis could be considered a limitation of the study, given that its alternative, RNA-seq, does not rely on predefined probe sequences. RNA-seq can detect signals across a wide range without limitation, theoretically providing more accurate estimates of gene expression levels. Nevertheless, in the present study, we were able to identify a relevant number of genes associated with joint ECM homeostasis and mediators of OA pathogenesis, shedding light on the potential mechanisms involved in the beneficial effects of CGH-NC and CGH treatments.

Conclusions

The results obtained in the present study suggest that both CGH-NC and CGH have the capacity to modulate chondrocyte ECM remodelling through the modulation of mRNA levels of genes involved in the biosynthesis of ECM components (CS and aggrecan) and the inhibition of proteolytic enzymes synthesis. The modulation of these processes could be potential mechanisms of action for both treatments and may account for their beneficial effects on various articular cartilage parameters (such as chondrocyte density or collagen degradation), as well as on cartilage and subchondral bone structures and MRI cartilage biomarkers. Finally, microarray data suggested that the modulation of the mTOR signalling pathway could be a potential mechanism underlying the greater effects observed after supplementation with the GAGs combination with NC, compared to GAGs alone. This includes greater improvement in MRI biomarkers of the articular cartilage and a higher content of hyaluronic acid in the synovial fluid.

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions
R.M-C., S.F., F.P. and A.C. (Antoni Caimari) designed the research; C.S., V.S., and C.I.S. designed and conducted the animal model; R.M-C., C.D-C., S.F., A.C. (Andrea Costa) conducted the research and analysed the data; R.M-C. and C.D-C. wrote the manuscript; R.M-C., C.D-C., S.F., A.C. (Andrea Costa), S.S., M.J.L-A., F.P., J.J.C., D.M-P., C.S., V.S., C.I.S, and A.C. (Antoni Caimari) reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability
Datasets analyzed during the current study will be made available from the corresponding author upon reasonable request.

Declarations

Ethical approval
All procedures were performed according to the European legislation on protection of animals and with the approval of the Local Government Animal Protection Ethics Committee (RD53/2013).

Consent for publication
Not applicable.

Competing interests
S.S. and D.M-P. are employees of Bioiberica S.A.U. The rest of the authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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